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CHANGES IN AND THE REGULATION OF PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE IN HUMAN GESTATION AND LABOUR.

BY

Fernando José Teixeira

C

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF PHYSIOLOGY

Edmonton, Alberta
Fall, 1993

UNIVERSITY OF ALBERTA

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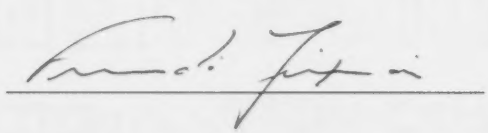

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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August 18, 1993.

To Caroline, Mom, Dad, Pia and Tino

Abstract

Prostaglandins are of primary importance in the initiation and maintenance of labour in women. A major intrauterine source of prostaglandins is the amnion, which synthesizes increased amounts of PGE_2 at term labour. Since prostaglandin endoperoxide H synthase (PGHS) catalyses the rate limiting step of prostaglandin synthesis from arachidonic acid, an investigation was made of the changes in amniotic PGHS specific activity during gestation and at term and pre-term labour. Also, the level of immunoreactive PGHS protein in the amnion was determined in order to study the mechanisms by which PGHS activity is regulated. PGHS activity, measured as the amount of PGE_2 produced by amnion microsomes under optimal substrate and cofactor conditions, was 2-fold higher ($p<0.05$) following spontaneous labour at term as compared to age-matched tissues collected before labour. Amnion samples from pre-term (<36 wks of gestation) non-labouring patients contained low levels of PGHS activity, which increased 4-fold after spontaneous pre-term labour ($p<0.05$). Cross-sectional analysis suggested that PGHS activity was low in the first and second trimesters of gestation, but increased abruptly before the expected time of term labour onset. Using an anti-ovine PGHS antibody recognizing both the constitutive and the inducible isoforms of the enzyme, PGHS protein was detected in all microsomal samples. However, there was no correlation between PGHS activity and the amount of total immunoreactive PGHS protein. Using an antibody specific for the inducible isoform of PGHS (PGHS-2), we detected immunoreactive protein in 9 of the 25 tissues examined, but found no correlation between PGHS activity and the amount of PGHS-2 protein. *In vitro* studies indicate that glucocorticoids inhibit PGHS activity, protein levels and *de novo* synthesis rates. In contrast, protein kinase C -activating phorbol esters increased PGHS activty, protein levels and *de novo* synthesis rates. These results suggest

that: 1) PGHS specific activity in the amnion increases sharply before the expected time of term labour; 2) amniotic PGHS activity increases at term and pre-term labour, 3) PGHS in the amnion is controlled by mechanisms which influence enzyme activity levels without concomitant changes in the concentration of immunoreactive enzyme protein, and 4) PGHS is positively and negatively regulated in the human by protein kinase C and glucocorticoids, respectively, suggesting that these effectors may regulate the enzyme *in vivo*.

Preface

This thesis has been prepared according to the guidelines regulating thesis preparation as put forth by the Faculty of Graduate Studies and Research. It has been written in a paper-based thesis format incorporating one first author publication and one second author publication by Fernando José Teixeira. Permission for inclusion of the second author publication (Chapter 3) has been granted and is found in Appendix 1. A version of Chapter 2 entitled "*In vivo* Changes in PGHS Activity and Protein levels" has been submitted to the Journal of Clinical Endocrinology and Metabolism under the manuscript title "Prostaglandin Endoperoxide H Synthase (PGHS) Activity and Immunoreactive PGHS-1 and PGHS-2 Levels in Human Amnion Throughout Gestation and at Labour." A version of Chapter 3 entitled "Regulation of PGHS *in vitro*" has been submitted and accepted for publication in the Journal of Reproduction and Fertility under the title "Regulation of Prostaglandin Endoperoxide H Synthase in the Amnion by Glucocorticoids and Activators of Protein Kinase C."

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List of Abbreviations

Ab:	antibody
ANOVA:	analysis of variance
ASA:	acetylsalicylic acid
BCIP:	5-bromo, 4-chloro, 3-indolyl phosphate
b.p.:	boiling point
Buffer H:	homogenisation buffer
Buffer I:	incubation buffer
C/S:	cesarean section
Da:	dalton
DDC; DTC:	dithiodicarbamic acid
EDTA:	ethylenediaminetetraacetic acid
GSH:	reduced glutathione
HEPES:	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
IC_{50}:	half-maximal inhibition
IgG:	immunoglobulin G
IL:	interleukin
IP_3:	inositol trisphosphate
ir:	immunoreactive
K_d:	dissociation constant
K_m:	Michaelis-Menten constant
M_r:	relative molecular weight
mRNA:	messenger ribonucleic acid
PBD:	phorbol-12,13-dibutyrate
PBSG:	phosphate buffered saline solution with gelatin

PDGF:	platelet-derived growth factor
PG:	prostaglandin
PGDH:	15 hydroxyprostaglandin dehydrogenase
PGEM:	PGE metabolite
PGFM:	13, 14-dihydro, 15-keto $PGF_{2\alpha}$
PGHS:	prostaglandin endoperoxide H synthase
pH:	negative log of hydrogen ion concentration
PKC:	protein kinase C
PLA_2:	phospholipase A_2
PLC:	phospholipase C
PMSF:	phenylmethyl sulfonyl fluoride
PsAF:	pseudoamniotic fluid
r:	coefficient of correlation
RIA:	radioimmunoassay
RU486:	17β-hydroxy-11β-(4-methylaminophenol)-17α-(prop-1-ynyl) estra-4,9-diene-3-one
SDS:	sodium dodecyl sulfate
S.E.M.:	standard error of the mean
SL:	spontaneous labour
TBS:	transfer blocking solution
TPA:	12-tetradecanoyl phorbol 13-acetate
Trp:	tryptophan
V_{max}:	maximal velocity
vol/vol; v/v:	volume per volume
$\bar{x}$:	mean

1. Introduction

1.1. General Overview

Parturition results from a complex interplay of maternal and fetal factors that act through a paracrine organ communication system. This system is composed on one side by the fetus and fetal compartment which consists of the amniotic fluid, the adjacent amnion and chorion tissue. The maternal contribution is the decidua and uterine myometrium. Changes in the hormonal milieu that signal fetal maturation traverse from the fetal to maternal sides to ultimately effect change(s) in the local maternal environment which are required for the initiation of labour. This thesis will examine the role of a group of locally produced factors called prostaglandins in the initiation of human labour. Specifically, the control of prostaglandin biosynthesis will be studied in relation to events associated with human labour onset.

1.2. Biochemistry of Prostaglandins

Prostaglandins (PG) and related eicosanoids are usually derived from the 20 carbon polyunsaturated fatty acid, 5,8,11,14-cis eicosatetraenoic acid (also called arachidonic acid), that is enzymatically converted to biologically active PG. They have come to be regarded as the final common mediator in many physiological processes including fetal organ maturation and parturition (Challis and Olson, 1988). Eicosanoid formation may be divided into three sequential steps: 1) the mobilization of arachidonic acid from cellular phospholipids; 2) the sequential conversion of liberated arachidonic acid to the prostaglandin endoperoxides, PGG_2 and PGH_2, respectively; and 3) either isomerization or reduction of PGH_2 to what are considered to be the biologically stable and active eicosanoids (Smith and Marnett, 1990). The levels of biologically active PGs can also be regulated by the rate of metabolism. The following sections will examine

the biochemistry of each of these three biosynthetic steps, as well as PG metabolism, in more detail.

1.2.1. Mobilization of Arachidonic Acid

The initiation of PG synthesis begins with the liberation of arachidonic acid from cellular stores. The release of arachidonic acid is accomplished by the action of specific acyl hydrolases called phospholipases. The two major species of phospholipases involved in PG generation are phospholipase A_2 (PLA_2) and phospholipase C (PLC). Each acts on specific groups of glycerophospholipids to effect the release of arachidonate through a cell-specific receptor mediated cascade system.

PLA_2 catalyses the hydrolysis of the *sn* -2 ester bonds of glycerophospholipids (Stryer, 1988). It is known to preferentially act on phosphatidylethanolamine, phosphatidylcholine and plasmalogens. Arachidonic acid is esterfied preferentially at the *sn* -2 position of phospholipids; therefore the action of PLA_2 results in the release of arachidonic acid (Olson, Zakar and Mitchell, 1993).

In comparison, the PLC pathway is involved in the indirect release of arachidonic acid. PLC catalyses the hydrolysis of the phosphodiester bond between diacylglycerol and the phosphate moiety of the head group of phospholipids. The action of PLC is preferential for phosphatidylinositol and results in the release of diacylglycerols and inositol phosphates. Diacylglycerol is then further metabolized to yield glycerol and free fatty acids, including arachidonic acid, through the sequential action of diacyl- and monoacylglycerol lipases. PLC is the rate-limiting step of this pathway (Olson, Zakar and Mitchell, 1993).

1.2.2. Metabolism of Arachidonic Acid

Once arachidonic acid is released, it can be acted upon by prostaglandin endoperoxide H synthase (PGHS) to produce the intermediate prostaglandins, PGG_2 and PGH_2. This enzyme has both a *bis* -dioxygenase (cyclooxygenase) activity, which catalyses PGG_2 formation, and a peroxidase activity which catalyses the reduction of PGG_2 to PGH_2. Both activities are found within the same protein molecule (Miyamoto *et al.*, 1976); however, the enzyme itself is known to exist as a homodimer. PGHS is a transmembrane glycoprotein with a relative molecular mass (M_r) of 70 kDa that is usually associated with the endoplasmic reticulum (Van der Ouderaa *et al.*, 1982) but has also been found on both the nuclear envelope and plasma membrane (Smith and Marnett, 1990). Although capable of metabolzing a variety of polyunsaturated fatty acid substrates (i.e. those containing at least three methylene interrupted *cis* -double bonds beginning at n-6) the most common *in vivo* substrate for PGHS is arachidonic acid. This enzyme has a K_m of approximately 2-10 μM for arachidonic acid (Marshall *et al.*, 1987; Smieja *et al.*, 1993). The intracellularly produced PGH_2 is then acted upon by specific synthases or isomerases to produce a variety of biologically active PGs. These metabolic steps are discussed in detail in the next section.

1.2.3. Metabolism of PGH_2

Most PG-forming cells produce only one type of eicosanoid because of the predominance of a single PGH_2 metabolizing enzyme. PG formation occurs through the action of a set of PG isomerases that produce PGs of the D,E, and I series (Smith and Marnett, 1990). As well, $PGF_{2\alpha}$ is produced via a two electron reduction of PGH_2 (DeWitt and Smith, 1983). In most tissues, PG levels are regulated by the rate of PG synthesis rather than the rate of metabolism (Olson,

Zakar and Mitchell, 1993). Once a PG is produced, it exits the cell, presumably via a carrier-mediated transport system (Lam et al., 1989), and exerts its effect on the same or neighbouring cell, most probably through a receptor-mediated signal transduction mechansim.

1.2.4. Metabolism of PG

The initial step in PG catabolism is catalysed by 15-hydroxyprostaglandin dehydrogenase (PGDH). It is a cytosolic enyzme with an M_r of 42 kDa (Thaler Dao *et al.*, 1974). As well, two isotypes of the enzyme have been identified; type I which is NAD^+ requiring and type II which is $NADP^+$ requiring (Myatt *et al.*, 1990). The products of PGDH action are 15-keto derivatives of PGs and are substantially decreased in their ability to elicit physiological responses (Mitchell, 1990). Once formed, the 15-keto PGs are then acted upon by a γ-reductase enzyme that reduces the double bond between carbon 13 and 14 to yield the 13,14-dihydro-15-keto PG metabolite (Myatt, 1990).

Given an understanding of the biochemistry and pathway of PG production and metabolism, an investigation can now be made of the physiological role of PGs in the processes of human labour onset. As well, the regulation of PG biosynthesis will be considered throughout gestation and during the process of labour onset, both term and pre-term.

1.3. Parturition

1.3.1. General Overview

The history and origins of PGs and their bioactivity are closely associated with the practice of obstetrics. Kurzrok and Lieb (1930) found that the addition of semen through intrauterine application sometimes caused contractions that expelled the semen. This effect was reproducible in early *in vitro* model systems

that employed uterine strips. It was concluded that human semen contained a factor that could stimulate uterine tissue to contract, and indeed in some situations, to relax. In the sixty years since these initial observations, there has been an explosion of knowledge regarding the importance of PGs as regulators of physiological processes. The involvement of eicosanoids, PGs in particular, in human pregnancy and parturition has been extensively studied. A commonly held view is PGs, produced within the intrauterine tissues (i.e. the fetal amnion and chorion and the maternal decidua), play a critical role in effecting the contractile stimulus(i) that act on the pregnant uterus (Wickland *et al.*, 1972) as well as mediating cervical ripening (MacKenzie and Embry, 1977) and membrane rupture (Challis and Olson, 1988). The role of PGs in the process of labour onset have centred on three lines of evidence: 1) During parturition, there is an increase in the concentration of PGE_2 and $PGF_{2\alpha}$ in amniotic fluid and of their metabolites in maternal plasma (Karim and Devlin, 1967); 2) Administration of drugs, such as indomethacin, that block PG synthesis suppress uterine activity and prolong pregnancy (Novy, 1974), and finally; 3) Myometrial contractility to exogenously added PG is observed throughout gestation and is found to increase at term (Thiery, 1979). Each of these observations will be discussed in the following sections.

1.3.2. PG Production at Labour

A considerable body of evidence indicates that increases in PG production are associated with the onset of labour. The predominant PGs involved in parturition, and more specifically myometrial contractile activity, are PGE_2 and $PGF_{2\alpha}$. Peripheral blood samples of women taken at the time of parturition show an increase in the concentration of 13, 14-dihydro 15-keto $PGF_{2\alpha}$ (PGFM), the metabolite of $PGF_{2\alpha}$ (Green *et al*, 1974). At the same time, the concentration of

PGE_2, PGFM, and $PGF_{2\alpha}$ increase in amniotic fluid samples (Bleasdale and Johnston, 1985). However, a critical caveat to these and similar studies is the dependence and timecourse of increased PG levels, measured either in the plasma or amniotic fluid, with respect to the onset and progression of labour. It is difficult to ascertain whether the increases observed are in fact preceding, and thus possibly causal, to the onset of labour; or whether they occur after the onset of labour and are therefore a consequence of labour or other factors initiating labour. For example, Weitz *et al.* (1986) found that plasma PGFM levels were elevated in women in preterm labour as compared to their gestational age-matched counterparts not in labour. From this, it would seem that PG levels, more specifically PGFM, rise in association with labour. However, in a similar study by Sellers *et al.* (1981), there was no difference in plasma PGFM levels between these two same groups of women. The discrepancies between these studies may be explained given the fact that other paracrine factors may be involved in PG generation. Romero *et al.* (1988) found that intraamniotic infections, which are significantly more common in preterm labour groups than in term labour populations, may be major determinants of elevated PG levels in both plasma and amniotic fluid. Therefore, the differences observed in these data may have resulted from differences in the inclusion-exclusion criteria for factors such as infection. The two most common tests for infection are histopathological studies which confirm the presence of intraamniotic infection by neutrophil invasion (Romero *et al.*, 1988) and amniotic fluid culture studies which identify bacterial flora present in the amniotic fluid and presumably in the fetal and maternal membranes (Romero *et al.*, 1988). The importance of identifying infection is critical in the examination of the effects of PGs on parturition since the products of inflammatory cells may themselves stimulate PG production.

1.3.3. Inhibition of PG Production

Indomethacin has been extensively used as a tocolytic agent because of its ability to irreversibly inhibit PG production. Numerous clinical trials (Zuckerman *et al.*, 1974; Wiqvist *et al.*, 1975) have demonstrated that preterm labour can be successfully delayed with indomethacin treatment. Although there is a wide variation in the protocols and mode of drug administration, most studies are able to demonstrate either a significant reduction in, or complete cessation of, uterine contractions. That these observed results could be correlated with decreased PG levels was first noted by Wiqvist *et al.* (1975) who measured plasma PGFM levels before and after indomethacin treatment. All patients showed a decrease in uterine contractions with some patients experiencing complete myometrial quiescence. Concomitantly, plasma PGFM levels fell markedly after indomethacin administration. Further, the daily administration of indomethacin was found to delay the onset of labour by up to three weeks. It was concluded from these data that PGs initiated myometrial contractions at labour. Care must be taken however in the interpretation of these results because of a lack of proper control groups required to properly establish a cause-effect relationship. For example, it has been shown that placebo subjects in preterm labour studies have a high rate of successful spontaneous cessation of uterine activity (Pritchard *et al.*, 1985). Nevertheless, the concentrations of PGs in amniotic fluid can be an important index of labour onset since abnormally low concentrations of PG are found in women who experience clinically delayed labour (Kierse *et al.*, 1977).

1.3.4. Myometrial Sensitivity to PG

Both the pregnant and non-pregnant myometrium respond to PG administration with increased uterine activity. During pregnancy, myometrial

sensitivity increases over the last third of gestation in humans (Thiery, 1979). These changes are important because during the early stages of pregnancy, myometrial quiescence must be maintained to prevent premature expulsion of the fetus. At the time of labour, coordinated myometrial activity must occur and be of sufficient strength to expel the mature fetus through the birth canal.

The smooth muscle of the myometrium is segregated into two muscle layers; an outer elongated smooth muscle layer and an inner circular smooth muscle layer. The cellular distribution of PG receptors differs only in the quantitative amounts of individual receptors since both layers have been characterized as having PGE, $PGF_{2\alpha}$, and PGI_2 receptors (Rao, 1990). In the non-pregnant uterus, circular and elongated myometrial layers have a similar number of PGE and $PGF_{2\alpha}$ binding sites with PGI_2 receptors predominating in elongated smooth muscle. The apparent equilibrium dissociation constant (K_d) for PG receptors have been found to be in the nanomolar range with the K_d for PGE receptors being approximately 2 nM and 10 nM for $PGF_{2\alpha}$ (Rao, 1990). Biochemical characterization of PGI_2 binding sites has been difficult because of the rapidity at which PGI_2 is converted to its metabolite, 6-keto $PGF_{1\alpha}$ (approximately 5-10 minutes) (Chegini and Rao, 1988). Binding studies therefore are hindered since the observed characteristics of the receptor may reflect the properties of the receptor with respect to metabolite occupancy as opposed to PGI_2 occupancy. If there is a change in the myometrial sensitivity near labour, it can be proposed that there is an increase in either the number of stimulatory PG receptors or their affinity to their physiological agonist in the absence of any endogenous changes in local PG levels. Indeed myometrial PGE_2 binding was found to be significantly higher in pregnant patients at term than in non-pregnant patients; however, PGE binding sites did not differ before or during labour at term (Giannopoulus *et al.*, 1985). The affinity of the receptor to the agonist did not

change in this study. The increased number of PGE binding sites resulted from an increase in cervical PGE receptors since the topographical distribution of these receptors from an area of high density in the fundus to an area of low density in the cervix was not observed in the pregnant uterus. (Rao, 1990).

1.3.5. Signal Transduction in the Myometrium

The signal transduction mechanisms of PG-mediated myometrial activity are complicated. To date, seven separate PG receptors have been described with four having excitatory effects and three inhibitory (Coleman, 1991). Excitatory PG receptors act through a G-protein to activate PLC which in turn selectively hydrolyzes phosphatidylinositol 4, 5-bisphosphate to generate two second messengers; inositol trisphosphate (IP_3) and diacylglycerol. IP_3 triggers the release of intracellular Ca^{2+} stores and causes a transient influx of Ca^{2+} (Reimer, 1990) which activates the myosin light chain kinases that are ultimately responsible for myometrial contractions.

PGs also play a role in gap junction formation and their action. Gap junctions are intercellular communication channels that allow the passage of ions and small proteins. Gap junctions open and close transiently in response to hormonal stimulation. In myometrium, their functional importance is to propagate action potentials throughout the muscle and coordinate the contractile response. Myometrial gap junctions increase prior to labour onset and these increases have been correlated with PG treatment (MacKenzie and Garfield, 1985). Therefore, another way in which stimulatory PGs, such as PGE_2 and $PGF_{2\alpha}$, may act is via an increase in the number of myometrial gap junctions. Further, the proposed action of inhibitory PG, such as PGI_2, may be through elevated intracellular cAMP levels which close gap junctions and thus prevent the propagation of action potentials. It has been shown that PGI_2-stimulated cAMP levels decrease

the movement of deoxyglucose through gap junctions (MacKenzie and Garfield, 1985). In the same way, the closing of gap junctions by PGI_2 may prevent the movement of ions necessary for the propagation of action potentials.

Myometrial activity during labour is a coordinated system in which increases in PGs act to elicit contractions not only through changes in $[Ca^{2+}]_i$ levels but also through gap junction formation and control. Increased myometrial activity may therefore result not only from increased PG levels, but also an increase in the number of receptors linking PGs to signal transduction systems.

1.3.6. Sites of PG production: Intrauterine Tissues

The preceding sections have documented the observed increases in PG levels at the time of labour onset and the evidence ascribing PGs an important role in labour initiation. The origin(s) of these elevated levels must now be considered. Because of their unique anatomical location and enzymatic capability, the fetal membranes and the maternal decidua are proposed to play a vital role in the elevated PG levels observed at labour. The amnion is an avascular fetal membrane that is bathed by the amniotic fluid on one side and is contiguous with the fetal chorion on the other. The maternally derived decidua is found adjacent to the fetal chorion on one side and the myometrium on the other. The relative contributions of each of these tissues to elevated PG levels at labour have been investigated by localizing and quantifying the distribution of PG synthesizing and metabolizing enzymes in these tissues.

1.3.7. Intrauterine Membrane Production of PG

The amnion produces predominantly PGE_2 and this output is found to increase with labour (Rose *et al.*, 1990). In fact, PGE_2 is produced almost to the exclusion of other eicosanoid products such as $PGF_{2\alpha}$ and thromboxane A_2

(Mitchell, Bibby and Hicks, 1978). Prostaglandin metabolism occurs via the action of PGDH which oxidizes the 15-hydroxyl group to a 15-keto group (Mitchell, 1990). The product of this reaction is then acted upon by PG-γ-reductase (Myatt, 1990). Metabolism of synthesized PGs in the amnion does not occur because of a diminished or absent PGDH activity in this membrane (Mitchell, Rogers and Wong, 1993). In contrast, chorionic metabolism of PGs occurs extensively due to its high PGDH activity; subsequently, release of PGFM and PGEM is elevated over all other tissues in the chorion (Cheung, 1990). Decidual production of PGE_2 and $PGF_{2\alpha}$ is less than that of the amnion with this membrane possessing some limited PGDH activity (Mitchell, Rogers and Wong, 1993). The uterus is also an important tissue for the production of PGs. The myometrium is the predominant tissue for prostacyclin (PGI_2) production (Barnford and Williams, 1980). PGI_2 differs from the other PGs discussed in that it has been shown to cause myometrial relaxation (Omini, Pasagiklian and Folco, 1978). In order for myometrial contractions to occur, the inhibitory effect of PGI_2 must be overcome. Casey and MacDonald (1986) propose that this inhibition is achieved via the glucocorticoid mediated production of lipocortin, a protein that diminishes the action of phospholipase A_2 and thus blocks the production of arachidonic acid. As a result, the production of myometrial contractions seems to result from a shift from a predominantly inhibitory hormonal state in which PGI_2 helps to maintain myometrial quiescence to a predominantly stimulatory hormonal state in which elevated PGE_2 and $PGF_{2\alpha}$ levels coupled to inhibited PGI_2 release create a paracrine environment conducive to myometrial activity.

1.3.8. PG Transfer to the Myometrium

Challis and Olson (1988) propose that the amnion is the major source of PG to the myometrium at the time of labour. PGE_2 produced by the amnion may

cross the chorion and decidua to reach the myometrium and elicit contractions. *In vitro* studies (Nakla *et al.*, 1986; Bennett *et al.*, 1988) have demonstrated the passage of ^{3}H-PGE_2 across full thickness human fetal membranes from the fetal to maternal side. The problematic situation that arises is whether PGE_2 is able to cross the metabolizing membranes of the chorion and decidua in sufficient quantities to elicit a viable physiological response. One proposed mechanism for PG transfer is the sequestering of PGDH in specific populations of chorion cells that correspond to non-vacuolated trophoblast cells, thereby allowing for transmembrane movement of unmetabolized PGE_2 (Challis *et al.*, 1990). At labour onset, the amnion, chorion and decidua not only produce PGE_2 in large amounts, but also $PGF_{2\alpha}$ and its metabolites. Such an observation may suggest the preferential enzymatic conversion of PGE_2 to $PGF_{2\alpha}$ by the action of PGE_2 9-keto reductase. However, Niesert, Christopherson and Korte (1986) found that the activity of this enzyme is unchanged with labour and that its activity is two orders of magnitude less than that of PGDH. It is therefore probable that the mechanism(s) governing increased PG production must occur at points in the cascade mechanisms that are common to both PGE_2 and $PGF_{2\alpha}$ production.

1.3.9. Regulation of PG by Oxytocin

The synthesis and regulation of oxytocin is complex (Hirst, Chibbar and Mitchell, 1993). In humans, the local paracrine production of oxytocin may be important not only in its function as a uterotonic agent but also as a mediator of PG production. For this thesis, consideration will be made only of the effects of oxytocin as they relate to myometrial contraction and PG synthesis and release. Before the onset of labour, the concentration of myometrial oxytocin receptors rises significantly (Fuchs *et al.*, 1984). This increase lowers the stimulation threshold for the initiation of uterine contractions by oxytocin to levels normally

observed in the plasma of third trimester women. As a result, oxytocin may have a physiological effect on myometrial activity without an actual increase in circulating oxytocin levels. The relationship between oxytocin and PGs was first demonstrated when the induction of labour by oxytocin was accompanied by increased concentrations of PGFM in peripheral plasma (Fuchs *et al.*, 1983). The level of interaction between oxytocin and PGs is the intrauterine tissues and myometrium since oxytocin receptors have been identified in these tissues (Fuchs, 1987). One proposed mode of interaction between oxytocin and PGs is by way of oxytocin-mediated arachidonate release. Whittaker *et al.* (1988) showed that stimulation of decidual cells by physiological concentrations of oxytocin increased the ^{3}H-arachidonate release purportedly through an effect on phospholipase A_2 or C. This increased substrate availability correlated to increased PG production in the decidua (Whittaker *et al.*, 1988). Moore *et al.* (1991) have also suggested that oxytocin-stimulated PG production is, in cultured amnion cells, mediated by protein kinase C activation. This is of particular interest since protein kinase C activation by synthetic phorbol esters in our laboratory results in an increase in the production of PGE_2 by human amnion cells (Zakar and Olson, 1988). It would appear that oxytocin acts to increase myometrial activity. Whether this effect is direct or indirect is debatable but there is evidence to show that oxytocin-induced myometrial activity is mediated through prostaglandin production specifically in the decidua (Whittaker *et al.*, 1988).

1.4. Regulation of PG Biosynthesis

1.4.1. Release of Arachidonic Acid

As previously mentioned, the natural precursor for PG biosynthesis in animal species is arachidonic acid. To be released from its intracellular lipid and phospholipid stores, arachidonate must be acted upon by specific acyl

hydrolyases called phospholipases. There is evidence to suggest that arachidonate release, as well as its metabolism, is regulated in gestational tissues at the time of labour. *In vivo* studies have shown that the concentration of arachidonic acid increases in amniotic fluid at labour (MacDonald *et al.*, 1974) and this increase is correlated with cervical dilatation (Kierse *et al.*, 1977). Further studies report that arachidonic acid levels decrease in chorioamnion following labour (Curbelo *et al.*, 1981). Such findings would lend support to the hypothesis that control of PG production rests at the level of substrate release. Sections 1.4.1.1 and 1.4.1.2 will examine the evidence implicating both PLA_2 and PLC in the regulation of PG biosynthesis in gestational tissues throughout gestation and labour.

1.4.1.1. PLA_2 Regulation of PG Synthesis

PLA_2 acts on the *sn* -2 ester bond of glycerophospholipids resulting in the production of free fatty acids and lysophospholipids. In fetal membranes, PLA_2 activity is selective for glycerophospholipids containing arachidonic acid at the *sn* -2 position (Schultz *et al.*, 1975; Okazaki *et al.*, 1978). Okazaki et al. (1981) examined changes in amniotic PLA_2 activity and found that it increased in late gestation (38-41 weeks) as compared to early gestation (13-17 weeks), suggesting an increase in PLA_2 levels with advancing gestation. There was no observed increase in PLA_2 activity in the chorionic and decidual membranes. The mechanisms responsible for elevated PLA_2 mRNA levels have been investigated with use of complementary deoxyribonucleic acid (cDNA) probes. High levels of PLA_2 mRNA were found in placental tissue with decreased messenger ribonucleic acid (mRNA) levels being reported in the amnion and chorion (Aitken, Rice and Brennecke, 1990). Further, PLA_2 mRNA levels increased in the placenta, but not in the fetal membranes, in association with

term labour onset. Using a polymerase chain reaction-derived 371 nucleotide sequence, Bennet *et al.*, (1991) found no labour-associated changes in PLA_2 mRNA levels in any of the gestational tissues. It would appear therefore that increases in PLA_2 activity and expression are not responsible for the observed increases in PG ouput from the amnion at the time of labour.

1.4.1.2. PLC Regulation of PG Synthesis

The release of substrate arachidonic acid may also be accomplished by the action of PLC which hydrolyses the phosphodiester bond between diacylglycerol and the phosphate moiety of the polar head group of inositol phospholipids (Bleasdale and Johnston, 1985). Phosphoinositol-specific PLC enzymes are catagorized into four major subgroups; α, β, γ, and δ with each subgroup arising from a distinct gene (Suh *et al.*, 1988). In the fetal membranes (Johnston *et al.*, 1990) and decidua (Sawaga *et al.*, 1982), diacylglycerol is further metabolized to produce glycerol and fatty acids, including arachidonic acid. Early studies by DiRenzo *et al.* (1981) found that fetal membranes and decidua contain high levels of PLC activity. Furthermore, Bala *et al.* (1990) have shown that PLC activity in the amnion is associated with a single 85 kDa species of PLC that is most likely a member of the δ subgroup. *In vivo* work has demonstrated six-fold increases in amnion PLC activity over gestation with two-fold increases being observed in decidual tissue (Okazaki *et al.*, 1981). There is no change in PLC activity following spontaneous labour onset (Bala *et al.*, 1990). Interestingly this same study also noted a loss of PLC activity in dispersed amnion cells following culture from d2 to d10. The loss of PLC was specific and would point to an acute regulation of the enzyme *in vivo* . To date, the agonists and intracellular mechanism(s) responsible for the maintenence of these PLC levels are unknown (Olson, Zakar and Mitchell, 1993). The use of *in vitro*

amnion cell culture systems do however implicate a role for oxytocin and purogenic agonists in regulating PLC. Moore *et al.* (1988) and Vander Kooy (1989) showed that oxytocin and adenosine triphosphate (ATP) caused rapid increases in inositol phosphate turnover with accompanying Ca^{2+} transients, indicating that these compounds activated PLC. Interestingly, only oxytocin, and not ATP, stimulated PGE_2 production, suggesting that the activation of PLC does not necessarily correlate with an observable increase in amnion PG production. As a result, consideration must be given to the regulatory factors involved in arachidonic acid metabolism to PGs. Central to this is a study of the enzyme responsible for arachidonic acid metabolism, PGHS.

1.4.2. Metabolism of Arachidonic Acid

1.4.2.1. Overview

The ability of a cell to synthesize PGs is dependent on the two step process of substrate release and metabolism. As discussed, PLA_2 and PLC are the major enzymes involved in the liberation of arachidonic acid from human gestational tissues. Although increases in both enzymes have been demonstrated throughout gestation, there is little evidence to correlate the observed increases in amniotic and plasma PG levels at term labour with increases in either PLA_2 or PLC activity.

1.4.2.2. Catalytic Properties of PGHS

PGHS catalyses the metabolism of arachidonic acid to the intermediate PG, PGG_2 and PGH_2, through respective cyclooxygenase and peroxidase reactions. In the Hamburg and Samuelsson (1967) model of cyclooxygenase action, PGHS holds the substrate molecule in a conformational position so that a kink develops between the C-9/C-10 single bond. A carbon radical is then

produced by the extraction of the *pro* -S hydrogen atom most proximal to the enzyme. An oxygen molecule is then incorporated at C-11, resulting in a serial cyclinization which in turn produces a bicyclic peroxide. Incorporation of the second oxygen molecule at the C-15 position is followed by a reduction reaction which ultimately produces PGG_2 (Smith and Marnett, 1990). The initial activation of the cyclooxygenase reaction is peroxide dependent (Smith and Lands, 1972) and may be inactivated by the action of non-steroidal anti-inflammatory drugs, such as acetylsalicylic acid (ASA). ASA has been shown to irreversibly acetylate a specific serine residue on the PGHS protein (DeWitt and Smith, 1988). The action of the acetylation is to sterically hinder the substrate-enzyme interaction required for the initiation of the cyclooxygenase reaction.

Once produced, PGG_2 is immediately acted upon by the peroxidase portion of the molecule to produce PGH_2. The peroxidase activity of PGHS catalyses the reduction of hydroxyperoxides to alcohols by the simultaneous removal of two electrons (Marnett *et al.*, 1988). This activity is known to preferentially reduce fatty acid hydroperoxides with decreased activity being exhibited towards other secondary and tertiary hydroperoxides (Ohki *et al.*, 1979). Reconstitution studies using PGHS apoprotein have also demonstrated a requirement for a heme prosthetic group for peroxidase function (Ogino *et al.*, 1978).

The co-localization of the two catalytic functions on PGHS leads to a unique system of enzyme control in which there are two distinct mechanisms of PGHS inactivation. The first involves an autoinactivation of the cyclooxygenase portion of the molecule. The rate of this 'suicide' inactivation is directly related to the rate of cyclooxygenase catalysis but is independent of the presence of the newly formed product (Smith and Lands, 1972). Biochemical studies show that each mole of purified enzyme can produce approximately 1300 moles of PGG_2

before cessation of cyclooxygenase activity (Kulmacz and Lands, 1985). The mechanism of autoinactivation may also involve an unstable cyclooxygenase protein intermediate which rearranges irreversibly to an inactivated cyclooxygenase without affecting peroxidase activity (Smith and Marnett, 1990). The second mode of enzyme inactivation results when there is a local accumulation of hydroperoxides around the PGHS molecule (Markey *et al.*, 1987). This buildup leads to a change in the heme spectrum and involves modification of the heme group. Since both catalytic sites require heme, there is a concomittant loss of total PGHS activity (Smith and Marnett, 1990).

1.4.2.3. Structural Properties of PGHS

PGHS purified from ovine seminal vesicles has an M_r of approximately 72 kDa as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Van der Ouderaa *et al.*, 1979). In comparison, the expected molecular mass, as determined from the primary peptide sequence, is approximately 65.5 kDa (DeWitt and Smith, 1988) with the difference between the two being accounted for by oligosaccharide attachment to the protein subunit (Van der Ouderaa *et al.*, 1977). The deduced amino acid sequences have been derived from cDNA's cloned from mice (DeWitt *et al.*, 1990), sheep (DeWitt and Smith, 1988), and humans (Yokoyama and Tanabe, 1989). There is an 88% sequence homology between the sheep and mouse protein and a 91% sequence homology between the sheep and human enzymes (Smith and Marnett, 1990). The majority of sequence differences are observed at the C- and N-terminal regions of the enzyme. This may be of primary importance because variations in the N-terminus region of PGHS may affect both the post-translational modification of the enzyme and its cellular localization.

The presence of a signal peptide region on PGHS would predict its

localization to the membranes of the cell. However, hydrophobicity profiles and hydrophobic moment studies do not reveal any obvious transmembrane domains (DeWitt *et al.*, 1990). As well, recent work by Dvorak *et al.* (1992) has shown PGHS to be associated with non-membrane bound lipid bodies within human mast cells, alveolar macrophages, type II pneumocytes and neutrophils. Taken together, these studies would suggest that PGHS may be present in the soluble fraction of cell homogenates.

Recent work in the fields of reproductive biology and immunology have expanded the breadth of knowledge concerning the structural and functional aspects of PGHS and its regulation by the discovery of a novel inducible isoform of PGHS, or PGHS-2. Section 1.4.2.4 will examine the characteristics of both PGHS-1 and PGHS-2.

1.4.2.4. Isoforms of PGHS

To date, two isoforms of PGHS have been identified; one which is apparently constitutively expressed (PGHS-1) and one whose levels increase transiently in response to agonist stimulation (PGHS-2) (Sirois, Simmons and Richards, 1992; Masferrer *et al.*, 1992; O'Sullivan *et al.*, 1992). Although both isoforms have identical cyclooxygenase and peroxidase catalytic actions and activities (Otto *et al.*, 1992), amino acid sequencing studies reveal only a 61% homology between PGHS-1 and PGHS-2 (Yokoyama and Tanabe, 1989). Molecular studies have also shown that the human inducible isoform is translated from a 4.0-4.8 kB mRNA (Hla *et al.*, 1992), while the constitutive isoform is translated from a 2.8 kB mRNA (DeWitt and Smith 1988). In the mouse (Wen *et al.*, 1993) and chicken (Xie *et al.*, 1993), these individual genes are known to arise from separate genomic loci; PGHS-1 is found on chromosome 2 while PGHS-2 is localized to chromosome 1. As yet, the human counterparts of the

PGHS-1 and PGHS-2 genes have not been mapped.

The observation that PGHS-2 is induced in response to agonist stimulation would suggest the presence of specific reponse elements in the upstream promoter region of PGHS-2. Indeed studies in the chicken show that the 1.6 kB promoter region for PGHS-2 contains a TATA box and a variety of enhancer elements including a serum response element (SRE), activating protein (AP)-1 and -2 sites and several signal protein (SP-1) sites (Xie *et al.*, 1993). While these studies have not been repeated in the human, work on the 5' flanking region of PGHS-1 in the human have been done. Wang *et al.* (1993) demonstrated that this region contains multiple transcription start sites in the absence of canonical TATA boxes. Their observations suggest that PGHS-1 has the characteristics of a housekeeping gene and thus is not rapidly inducible.

Along with molecular studies in the mouse and chicken, there have been several reports of PGHS-2 induction in the human using *in vitro* paradigms. Han *et al.* (1990), using $p60^{vsrc}$-transformed 3T3 fibroblasts, were able to stimulate PGHS-2 gene expression within two hours of agonist stimulation with elevations over baseline being observed fifteen hours post-stimulation. Using confluent cultured amnion cells, Mitchell *et al.* (1992) were able to induce PGHS-2 mRNA following sixteen hour stimulation with interleukin 1-β. *In vivo* studies by Bennett *et al.* (1992) showed that term spontaneous labour onset is correlated with an increase in PGHS-1 mRNA in human amnion and placenta. To date, no data is available as to the *in vivo* protein levels of either PGHS-1 or PGHS-2 in human gestational tissues.

1.4.2.5. PGHS *in vivo*

Prior to PGHS isoform distinction and molecular analysis, many investigators had centred their work on the regulation of PGHS by examining the

changes in PGHS enzyme activity and protein distribution. Because of identical enzymatic properties and similar protein structure, measurements of PGHS activity and immunocytochemical studies most probably represent both PGHS-1 and PGHS-2 levels. In humans, immunocytochemical techniques have been employed to localize PGHS within both the intrauterine and intracellular compartment. Bryant-Greenwood *et al.* (1987) and Price *et al.* (1989) detected PGHS in all fetal and maternal membranes with the greatest intensity of staining being observed in the decidua. Within the epithelial layer of the amnion, there was a hetrogeneous distribution of PGHS with the adjacent subepithelial connective layer staining for PGHS mainly in the cytoplasmic fraction of fibroblast-like cells. No change in the intensity of staining was detected with gestation and labour in these membranes.

Although immunocytochemical studies are able to localize and assess the presence of PGHS in intrauterine tissues, they do not convey information as to the activity of PGHS within these membranes. Okazaki *et al.* (1981) and Smieja *et al.* (1993a) performed studies to determine the changes in PGHS activity at term labour and found that enzyme activity increased in the amnion. The relative difficulty in obtaining early trimester tissue has necessitated the adaptation of animal models to describe changes in PGHS activity throughout gestation. Elliot *et al.* (1984) found a linear 10-fold increase in amniotic PG synthesis over the last third of gestation in rabbits while in sheep the pattern of increased activity is characterized by a dramatic increase in cotyledonary PGHS activity in the final third of gestation (Rice *et al.*, 1988). These data point to species-specific changes in PGHS activity. Changes in PGHS activity throughout human gestation have not been described.

1.4.2.6. *In vivo* Regulation of PGHS

The *in vivo* mechanisms regulating increases in PGHS activity are not clearly defined. Smieja *et al.* (1993a) found that increased PGHS activity was associated with increases in the V_{max} of the enzyme rather than an increase in its K_m, indicating that increases in PGHS activity are correlated to increases in enzyme catalytic rates rather than increases in the enzyme's affinity for substrate. Two plausible explanations for this may be either a protein phosphorylation mechanism by which PGHS is activated or conversely, an increase in the overall amount of active enzyme present. Indirect studies by Gaffney *et al.* (1990) and Zakar *et al.* (1992) have explored the latter of these possibilities. Gaffney *et al.* (1990) were able to demonstrate an increase in the rate of recovery of PGE_2 synthetic capacity following irreversible inactivation of PGHS in both dispersed chorioamnion and placental cells obtained prior to labour as compared to cells prepared from post-labour tissue. These data point to an elevated rate of PGHS activity before labour at a time when PGHS activity and PG levels are increasing. The apparent increase in PGHS synthesis may arise from increases in gene transcription, increased mRNA stability, increased protein translation, PGHS isoform induction, or decreased enzyme inactivation or degradation.

1.4.2.7. *In vitro* Regulation of PGHS

The elucidation of PGHS activity regulation in human intrauterine tissues requires *in vitro* protocol systems. Amnion cell culture studies have shown PGHS activity to be positively regulated by epidermal growth factor (EGF) and activators of protein kinase C. In confluent amnion cell preparations, EGF is known to stimulate increased PGE_2 output through increases in both PGHS activity and *de novo* protein levels (Casey, Korte and MacDonald, 1988). Similarly, Zakar and Olson (1988a) demonstrated the EGF-dependent increase

to be protein synthesis dependent and lasting up to five hours. The mechanism by which EGF acts is unresolved. Kniss *et al.* (1990), using WISH cells (an amnion cell line) was able to block EGF's effects by the addition of the protein kinase C inhibitor staurosporine. Further, EGF's effect was augmented by the addition of the synthetic phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). Interestingly, TPA alone had no effect on PGE_2 output.

Studies investigating the role of protein kinase C directly have also been performed. Lytton and Mitchell (1988) stimulated PGE_2 output with TPA in the presence of exogenous arachidonic acid. Zakar and Olson (1988b) found this effect to be both RNA and protein synthesis dependent. Further, Smieja, Zakar and Olson (1993b) showed that protein kinase C increased the V_{max} of PGHS, again suggesting either an increase in the overall PGHS levels or an increase in enzymatic catalytic rates.

Glucocorticoids have long been associated with fetal organ maturation and the initiation of birth (Challis and Olson, 1988). In the sheep (the most common experimental model used to study labour initiation), rising fetal cortisol levels are presumed to be the trigger of labour onset by its action on the maturation of the hypothalamic-pituitary-adrenal (HPA) axis. Although there are no significant changes in circulating maternal plasma cortisol levels, intrauterine paracrine effects on PG production rates have been noted. In the human, amniotic cortisol levels rise before the onset of labour (Murphy, Patrick and Denton, 1975). As well, the amnion and chorion possess high-affinity glucocorticoid binding sites with the characteristics of steroid hormone receptors (López Bernal and Turnbull, 1985). In confluent amnion cells, glucocorticoids stimulate PGE_2 production (Potestio, Zakar and Olson, 1988). This effect is elicited by an increase in PGHS activity (Smieja, Zakar and Olson, 1993b). In contrast, the effects of glucocorticoids in dispersed cell systems suggest an inhibitory role in PG

production. Gibb and Lavoie (1990) found PGE_2 output in dispersed amnion cells to be inhibited by the addition of dexamethasone, a synthetic glucocorticoid that acts specifically on cortisol binding sites. Since the responses of freshly isolated amnion may reflect the *in vivo* responsiveness of amnion tissue more closely than the responses obtained from confluent cultured amnion cells, a tissue incubation system should be employed to study the effects of glucocorticoids on the amnion. To date, the role of glucocorticoids in affecting changes in PGHS activity and protein levels have not been described in this model system.

1.5. Rationale

Increased PG levels within the intrauterine compartment play a pivotal role in the processes leading up to labour onset in women. The fetal amnion produces increased amounts of PGE_2 in association with labour. PGE_2 is thought to stimulate myometrial contractions as well as eliciting cervical remodeling and membrane rupture. These events are required for the successful expulsion of the fetus from its *in utero* environment. The biochemical processes in PG production involve both the release and metabolism of arachidonic acid. The enzymes PLA_2 and PLC are involved in arachidonate mobilization in the amnion. While the activities of these enzymes increase over gestation, neither is apparently responsible for the increased plasma and amniotic PG levels observed at the time of labour. Further, the amnion has been shown to have little or no capacity to metabolize PGs because of the absence of PGDH within this tissue. Hence altered activity of the arachidonic acid metabolizing enzyme, PGHS, must be considered in the overall regulation of PG biosynthesis at labour.

The proximity of the fetal amnion to the amniotic fluid compartment and maternal decidua and the absence of vascularization of the amnion would

suggest a role for locally produced factors in regulating amnion PG production. It is likely that these factors act at the level of PGHS to control the synthesis of PGE_2 by the amnion. Cortisol and endogenous activators of protein kinase C, such as diacylglycerol, are known effectors of increased PG production in the amnion. Therefore, the overall theme of this thesis is to investigate the *in vivo* and *in vitro* mechanisms by which PGHS may act to regulate PG production, both throughout gestation and at labour.

1.6 Hypotheses

The specific hypotheses tested within this thesis are:

1. Increases in PGHS activity occur before the onset of term labour in the human amnion.

2. Increased in PGHS activity occur before the onset of preterm labour in the human amnion.

3. Increases in PGHS activity are correlated with increases in the amount of immunoreactive PGHS protein present within the human amnion.

4. PGHS activity and immunoreactive PGHS protein levels are inhibited by the action of glucocorticoids and stimulated by activators of protein kinase C in the human amnion.

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2. *In vivo* Changes in PGHS Activity and Protein Levels

2.1. Introduction

Prostaglandins (PG) play an important role in the initiation and maintenance of labour in a variety of species, including humans (Bleasdale and DiRenzo, 1989). PGs produced by the maternal decidua and fetal amnion and chorion (Mitchell, 1988) may contribute to increased myometrial activity, cervical remodeling and membrane rupture (Challis and Olson, 1988). Although there is some uncertainty as to the relative contribution and exact physiological role of the various PGs generated in the intrauterine tissues, the consensus is that labour is associated with increased amnion PGE_2 production (Okazaki *et al.*, 1981a). The cascade mechanism responsible for PGE_2 production in the amnion includes the release and oxygenative metabolism of arachidonic acid (Olson *et al.*, 1993). Arachidonate is released from phospholipids by phospholipase (PL) A_2 or PLC, which are activated presumably through receptor mediated mechanisms (Bleasdale and Johnston, 1985). Although the levels of PLA_2 and PLC activity increase in the fetal membranes in mid-gestation, PG production remains low; as well, there is no increase in PLA_2 and PLC activity levels to accompany the observed increase in amnion PGE_2 output at term labour (Okazaki *et al.*, 1981b).

Prostaglandin endoperoxide H synthase [EC 1.14.99.1] (PGHS) catalyses the metabolism of free arachidonate to the bioactive intermediate prostaglandins PGG_2 and PGH_2 through the action of separate cyclooxygenase and hydroperoxidase catalytic sites respectively (Smith and Marnett, 1991). Prostaglandin E_2 is produced via the rearrangement of PGH_2 by PGH_2-E_2 isomerase (Smith, 1989). The conversion of arachidonic acid to PG endoperoxides is a rate limiting and committed step of prostaglandin biosynthesis. The control of PG production throughout gestation and at labour

must therefore incorporate an understanding of the mechanisms by which PGHS is regulated *in vivo*. In the human amnion, PGHS activity has been shown to increase during term labour onset (Okazaki *et al.*, 1981a; Smieja *et al.*, 1993) while animal studies have shown that PGHS activity increases throughout gestation (Elliot *et al.*, 1984; Noden *et al.*, 1982) and at labour (Rice, Wong and Thorburn, 1988). There is little data available as to the properties and the regulation of PGHS in human gestational tissues during pregnancy and at term or pre-term labour. Activators of protein kinase C and glucocorticoids have been shown recently to modulate PGHS activity and protein levels in post-labour human amnion *in vitro* (Zakar *et al.*,1993); however, the significance of these findings with respect to the changes of PGHS activity at labour remains to be established. Furthermore, PGHS regulation may also involve the differential expression of PGHS isoforms. To date, two isoforms of PGHS have been identified; one which is apparently constituitively expressed (PGHS-1), and one whose levels increase transiently in response to agonist stimulation (PGHS-2) (Sirois, Simmons and Richards, 1992; Masferrer *et al.*, 1992; O'Sullivan *et al.*, 1992). In gestational tissues, increases in PGHS expression have been identified with respect to term labour onset (Bennet, Henderson and Moore, 1992) and cytokine stimulation (Mitchell *et al.*, 1992). The changes in PGHS isoform composition and levels with labour and gestation in the amnion have not been described.

The present study was designed to examine the changes in PGHS activity and immunoreactive (ir) protein levels with advancing gestation and labour in human amnion. We hypothesized that increased PGHS activity is associated with both pre-term and term labour in the human. Increased enzyme activity may involve an increase in PGHS levels as a result of an increased rate of protein

synthesis. Therefore we also investigated the possibility that changes in PGHS activity values would be correlated to changes in the immunoreactive levels of the enzyme.

2.2. Materials and Methods

Materials

The following materials were purchased from Sigma Chemical Co. (St. Louis, MO): leupeptin, phenylmethylsulfonly fluoride (PMSF), diethyldithiocarbamic acid (DDC) and tryptophan (Trp). [5,6,8,11,12,14,15-N-^{3}H] PGE_2 (specific activity, 140 Ci/mmol), an anti-rabbit IgG horseradish peroxidase conjugate and reagents used in enhanced chemiluminescence were obtained from Amersham Canada (Oakville, ON). PGHS purified from sheep seminal vesicles and a rabbit polyclonal antibody (Ab) raised against sheep seminal vesicle PGHS were purchased from Cayman Chemical (catalogue #160102, Ann Arbor, MI). Chicken recombinant mitogen-inducible prostaglandin H synthase (PGHS-2) was obtained from Oxford Biomedical Research (Oxford, MI). Arachidonic acid and reduced glutathione (GSH) were bought from Nu-Chek Preparations (Elysian, MN) and Boehringer Mannheim Canada (Laval, Quebec) respectively. Sep-PaK C_{18} cartridges were products of Waters-Millipore (Milford, MA). Anti-rabbit IgG-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) were supplied by Promega (Madison, WI.). Reagents for SDS-polyacrylamide gel electrophoresis and electrophoretic transfer materials were obtained from Fisher Scientific (Ottawa, ON). Prestained SDS-polyacrylamide gel electrophoresis standards were obtained from Bio-Rad Canada (Mississauga, ON). All other chemicals were of analytical (ACS) purity.

Tissue Collection

All placentas were from singleton pregnancies were collected immediately upon delivery and placed in ice cold physiological saline. Reflected amnion was isolated from other intrauterine tissues and placed in 30 ml of ice cold pseudoamniotic fluid (PsAF) (Schwartz *et al.*, 1977). The isolated amnion was cleaned of blood clots and cut into 5 cm strips. Amnion strips were rinsed 3 times in petri dishes containing 10 ml PsAF and frozen in liquid nitrogen until further processing. All frozen tissues were stored at -70°C and processed within three weeks of collection. The collected tissue samples were examined histologically and only those free of infection, as determined by neutrophil invasion, were used in the studies. The tissue collection protocol was approved by the University of Alberta Ethics Review Committee.

Patient Data

Preterm amnion samples were collected following either spontaneous labour (SL) or cesarean section (C/S) performed in response to pregnancy-induced hypertension, placenta previa or fetal distress. The gestational ages for these tissues ranged from 22 to 36 weeks gestation as confirmed by crown-to-rump lengths using Doppler ultrasound. The amnion sample collected prior to 22 weeks gestation were obtained following elective termination of pregnancy and were included in the C/S group. These tissues were kindly donated by Dr. Bela Resch, Szeged, Hungary. Term amnion tissue was collected from either spontaneous labour deliveries or elective cesarean section. Term amnion samples had a gestational age range of 37-41 weeks with the mean gestational ages being 38.5 and 38.7 weeks for the C/S and SL groups, respectively. There was no significant difference in the length of gestation between the C/S and SL

group. The fetal and maternal outcomes from tissues collected at term were normal at the time of hospital discharge.

Microsomal Preparation

To study the changes in PGHS enzyme activity, we have adapted a previously described PGHS enzyme assay (Smieja *et al.*, 1993). Briefly, 2 g of frozen amnion tissue was crushed in mortars pre-cooled with liquid nitrogen and placed in 20 ml of homogenization buffer (50 mM Tris HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.25 M sucrose) containing 10 µg/ml leupeptin and 1 mM PMSF. Samples were then homogenised with a Brinkman Polytron homogeniser fitted with a medium sized generator for 3x30 s at setting 5 and 3x30 s at setting 6. The homogenate was centrifuged at 1000x g for 15 min at 4°C and the supernatant collected. A crude particulate fraction was prepared from the 1000 g supernatant by ultracentrifugation at 105 000 *g* for 60 min at 4°C. The resulting pellet was re-suspended in 1.5 ml of re-suspension buffer (50 mM Tris HCl, 2 mM EDTA, 1 mM DDC, 0.5 mg/ml Trp) by homogenising manually with a Potter-Elvehjem tissue grinder. This suspension, containing microsomes, was used as the enzyme preparation in the determination of PGHS activity.

Incubations

All microsomal incubations were carried out in a 37°C water bath for 4 min in acid washed 12x75 mm test tubes in the presence of optimal cofactor concentrations (1 mM Trp, 1 mM GSH) (Smieja *et al.*, 1993). Preliminary experiments indicated that there was no statistical difference between the calculated V_{max} determined by an arachidonate concentration range and the specific activity of PGHS at 20 µM arachidonate, a concentration which is close

to saturation (appendix 3, Fig. A3-1). Consequently, we have employed an assay system using 20 μM arachidonate to quantitate PGHS enzyme activity. Each microsomal preparation was assayed at three protein concentrations in triplicate in order to ascertain the linear relationship between enzyme activity and the protein content of the reaction mixture. Reactions were started by the addition of 50 μl of the prepared microsomes to the incubation mixture. Incubations were stopped by adding 4 ml of citrate buffer (50 mM, pH 3.0) containing 15% (v/v) ethanol. All samples were frozen and extracted within 72 h of incubation.

Extraction and RIA

Prostaglandin E_2 produced was extracted by using Sep-Pak C_{18} cartridges (Powell, 1982). The extract was dried under a stream of nitrogen gas and the residue redissolved in phosphate buffered saline. This procedure consistently yielded recovery levels of 90% or greater and correction was made for individual extraction losses. Quantitation of PGE_2 was performed using a specific radioimmunoassay (Olson *et al.*, 1984).

Protein Assay

Microsomal protein levels were determined by the Bradford technique (Bradford, 1976) using bovine serum albumin as standard and a dye solution purchased from Bio-Rad as an indicator.

Immunoblotting Procedures

Microsomal amnion samples were prepared as described above and diluted 1:1 with loading buffer (Tris 0.2 M, SDS 2%, 2-mercaptoethanol 5%) (Laemeli, 1970). All samples were heated at 95°C for 15 min prior to

electrophoretic separation. Samples were then separated on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes (Towbin, Staehelin and Gordon, 1979). PGHS immunoreactivity was detected by incubation with either a rabbit polyclonal Ab raised against ovine PGHS (Cayman Biochemical) or a rabbit polyclonal Ab raised against the unique 17 amino acid carboxy terminus of PGHS-2. This Ab was kindly donated by Dr. D. DeWitt, Michigan State University (O'Sullivan *et al.*, 1992). Antibody binding for PGHS was visualized using an anti-rabbit IgG-alkaline phosphatase conjugate and a Problot alkaline phosphatase detection system (Promega). PGHS-2 was visualized using an anti-rabbit IgG horseradish peroxidase conjugate and an enhanced chemilumenesence detection system (Amersham). Densitometric analyses were performed using an LKB Ultrascan (Pharmacia).

Statistical Analyses

The sample size for these studies was determined in preliminary experiments. These experiments indicated that the coefficient of variation of PGHS specific activity was 30%. The two tailed test formula, $n=2(Z_{\alpha/2}+Z_{\beta})^2(\sigma/\delta)^2$, was used to determine the number of patients required for each study group. The assumptions were that $\alpha=0.05$, $\beta=0.2$, σ=interassay standard deviation (determined above) and that the minimal reasonable difference in PGHS specific activity values that would suggest a biological difference in patient groups was 2.0σ (defined as δ). It was determined that the minimum number of patients needed per study group was 8. Interative analysis using actual PGHS activity results confirmed this sample size.

Enzyme activity values are expressed as pg PGE_2/µg protein/min. Results are expressed as the $\bar{x}$ ± S.E.M. The number of patients in each study

group is described in each figure. The data in figures 1 and 2 were analysed using Student's t-test with significance being achieved at $p<0.05$. Linear (Figs. 2-5 and 2-7) and polynomial (Fig. 2-3) regression analyses were performed using the Minitab® statistical package (State College, PA).

2.3. Results

Labour-associated changes in amniotic PGHS were examined by comparing the PGHS activity of term pre-labour C/S amnion to that of term post-labour SL tissue. PGHS specific activity was 18.2 ± 3.7 pg PGE_2/μg protein/min ($\bar{x}$ ± S.E.M.; n=19) in the pre-labour, C/S group. Following spontaneous labour and delivery, this value increased significantly ($p<0.05$) to 38.9 ± 6.0 pg PGE_2/μg protein/min (n=19; Fig. 2-1). These data demonstrate that PGHS specific activity increases with term labour. Increases in PGHS specific activity were also observed with pre-term labour onset (Fig. 2-2). The specific activity of PGHS increased from 5.9 ± 1.8 pg PGE_2/μg protein/min (n=9) in the pre-term, not in labour (C/S) group to 28.3 ± 6.8 pg PGE_2/μg protein/min (n=10) ($p<0.05$) in the pre-term SL group. All tissues collected from the pre-term SL group were histologically confirmed to be free of infection and thus termed idiopathic deliveries.

To investigate the gestational age dependent increases in PGHS specific activity, non-laboured amnion samples were analysed by plotting the enzyme activity levels versus the gestational age of the tissues at the time of obstetrical intervention (Fig. 2-3). Cross-sectional analysis of these data indicated that the enzyme activity remained low (< 10 pg PGE_2/μg protein/min) in the first and second trimesters of pregnancy with a marked increase in the final third of gestation immediately prior to the expected time of labour onset. Regression

analysis showed that the increase in PGHS specific activity across gestation was best described by the equation $y=1.64\ x-6.69x10^{-2}\ x^2+2.4x10^{-5}\ x^3$ ($p<0.05$).

The amount of PGHS protein in the microsomal fractions was assessed by immunoblotting. Using a polyclonal Ab raised against sheep seminal vesicle PGHS, we detected an immunoreactive band co-migrating with the purified sheep seminal vesicle PGHS standard (70 kDa) in all amnion samples. Figure 2-4 shows a representative immunoblot containing samples from 6 amnion microsomal preparations. Compared to the immunoreactivity of the standard, the amount of PGHS protein in the preparations was estimated to range between 0.1 and 10 fmol/μg microsomal protein. In addition, a lower molecular weight (50-53 kDa) band was observed in all samples examined. Immunocompetition studies using an excess amount of purified ovine PGHS confirmed that the 70 kDa band corresponded to PGHS. Immunoreaction with the 50-53 kDa material was also blocked by excess PGHS indicating that this band most likely represented a proteolytic degradation product of the enzyme (Teixeira *et al.*, 1993). Correlational analysis of PGHS enzyme activity and immunoreactivity, expressed as the intensity of the 70 kDa band, is shown in figure 2-5. As illustrated, no correlation was found between PGHS activity and the amount of immunoreactive PGHS ($y= 0.96x+ 19.81$; $r= 0.04$; $p>0.05$).

To investigate the possibility that changes in PGHS specific activity may have resulted from a selective increase of PGHS-2, the inducible isoform of the enzyme, the microsomal samples were analysed using an Ab specific for PGHS-2. This Ab, detected a 70 kDa band on immunoblots containing recombinant PGHS-2 (Fig. 2-6). Immunoreactive bands co-migrating with the PGHS-2 standard were observed in 9 of 25 tissues studied. Where detected, the amount

of immunoreactive PGHS material ranged between 100 and 750 fmol/μg microsomal protein, as compared to the immunoreactivity of recombinant PGHS-2. Regression analysis demonstrated a lack of significant correlation between the amount of immunoreactive PGHS-2 and PGHS enzyme activity levels ($y= 1.71x+ 19.79$; $r= 0.11$; $p>0.05$) (Fig. 2-7). The anti-PGHS-2 Ab did not react with the purified ovine PGHS standard suggesting that this preparation consisted mostly or entirely of PGHS-1. On the other hand, the anti-ovine PGHS Ab used in the experiments presented in Figures 2-4 and 2-5 recognized the PGHS-2 standard, indicating that this immunoreagent detected both isoforms of the enzyme (Fig. 2-8). Consequently, results obtained with this Ab are indicative of the levels of total PGHS-1 and PGHS-2 immunoreactive enzyme.

2.4. Discussion

In the present study, we examined the changes in PGHS activity and immunoreactive levels in the human amnion throughout gestation and labour. The data demonstrate that PGHS specific activity increases 3-fold from 5.9 to 18.2 pg PGE_2/μg protein/minute over the final trimester of pregnancy before the onset of labour. Our results also demonstrate a further labour-dependent increase in PGHS specific activity. In the case of term labour onset, PGHS specific activity increased from 18.2 to 38.9 pg PGE_2/μg protein/minute. The present findings are consistent with those of Smieja *et al.* (1993) and Okazaki *et al.* (1981a) who reported respective 1.5- and 2.0-fold increases in PGHS activity with labour in human term amnion tissue. A significant ($p<0.05$) 4-fold increase in PGHS specific activity from 5.9 to 28.3 pg PGE_2/μg protein/minute was also observed in the case of idiopathic pre-term labour onset. Although the etiology of pre-term labour remains unclear (Romero *et al.*, 1988), our results suggest that a common mechanism associated with both pre-term and term labour may be an

increase in the activity of PGHS in the amnion.

Our study has also examined the changes in PGHS specific activity across gestation. PGHS activity is reported to increase in several species over gestation, however, the gestational profiles of these increases appear to be species-specific. Elliot *et al.* (1984) found a linear 10-fold increase in amnion PG synthesis over the last third of gestation in rabbits while in sheep, the pattern of increased enzyme activity is characterised by a dramatic increase in cotyledonary PGHS activity in the final third of gestation (Rice, Wong and Thorburn, 1988). Our results also indicate a significant ($p<0.05$) pre-parturient rise in PGHS specific activity immediately prior to the expected time of labour onset and are therefore consistent with the proposed hypothesis that increases in PG levels at term are caused, at least partially, by changes in the activity of PGHS.

Previous studies in our laboratory have shown that increases in PGHS activity at term labour are due to an increase in the V_{max} of PGHS rather than a change in its K_m (Smieja *et al.*, 1993). This would suggest either an increase in the catalytic activity of the existing enzyme or an increase in the amount of active enzyme present in a tissue. Indeed, Gaffney *et al.* (1990) have provided indirect evidence supporting this latter possibility by demonstrating an increase in the rate of recovery of PGE_2 synthetic capacity from exogenous arachidonate following irreversible inactivation of PGHS by aspirin in both dispersed chorioamnion and placental cells. These results point to an increased rate of PGHS synthesis prior to labour at a time when PGHS activity is also increasing. *In vitro* amnion cell culture studies have shown PGHS activity to be positively regulated by EGF and activators of protein kinase (Casey, Corte and MacDonald, 1988; Zakar and

Olson, 1988a; Zakar and Olson, 1988b). The role of glucocorticoids in controlling PGHS activity is at present unclear since their actions may be both stimulatory in amnion cell culture systems (Smieja, Zakar and Olson, 1993) and inhibitory in dispersed amnion cells (Gibb and Lavoie, 1990) and in tissue mince paradigms where glucocorticoids have recently been shown to inhibit the synthesis of PGHS (Zakar *et al.*, 1993).

Our study has attempted to characterize the *in vivo* regulation of PGHS by comparing the specific activity of the enzyme with the levels of irPGHS in the amnion tissue samples. In addition to the lack of a correlation between PGHS specific activity and irPGHS levels in individual patients, we also found no change in irPGHS levels with labour onset. Similar discrepancies in PGHS activity and immunoreactive levels have also been reported by DeWitt (1991) who described 2-3-fold increases in mitogen induced PGHS activity without concomitant increases in irPGHS levels in Swiss 3T3 fibroblasts. These results suggest that PGHS activity may be controlled by the rate of enzyme turnover and *de novo* PGHS synthesis. Increases in enzyme synthesis rates may be accompanied by an increased inactivation rate of PGHS so that the relative amount of active enzyme in a tissue increases without corresponding changes in the amount of immunoreactive protein. Such a system would not require any changes in the transcription rate of message for PGHS. The findings of Zakar *et al.* (1993) would suggest that *de novo* PGHS synthesis involves mechanisms which are not transcription dependent. However, Bennet, Henderson and Moore. (1992) have demonstrated increases in the 2.8 kB messenger ribonucleic acid (mRNA) for PGHS following labour onset in both amnion and placental tissue. Increases in PGHS mRNA levels may account for the large increases in PGHS activity levels observed at birth while gradual increases in PG and PGHS activity

levels, such as those observed throughout human gestation, may reflect regulatory mechanisms associated with changes in enzyme turnover rates.

A second mechanism that may regulate the activity of PGHS and thus PG production at labour and throughout gestation is the induction of the PGHS-2 isoform. While sharing similar catalytic and enzyme kinetic properties (Otto *et al.*, 1992), recent evidence suggests that PGHS-2 is differentially regulated. Wang *et al.* (Wang *et al.*, 1992), studied the upstream promoter region of PGHS-1 and found multiple transcription start sites in the absence of canonical TATA boxes. Their observations suggest that the PGHS-1 has the characteristics of a housekeeping gene. Although similar molecular studies have not been performed on the PGHS-2 gene, there is evidence to suggest that PGHS-2 expression involves an immediate-early response to specific agonist stimulation. Han *et al.* (1990) found that peak induction of PGHS-2 occurs within two hours of agonist stimulation and is found to return to baseline values approximately 15 h post-stimulation in $p60^{vsrc}$-transformed 3T3 fibroblasts. *In vivo*, stimulation of estradiol and FSH primed rat granulosa cells by hCG results in increased PGHS-2 gene expression by 4-5 h post-stimulation while protein levels were found to peak at about 6 h post-stimulation (Sirois, Simmons and Richards, 1992). The detection of PGHS-2 in only 9 of 25 samples is consistent with the transient expression of both the gene and protein. Increases in PGHS-2 may occur in the hours preceding the obstetrical intervention or labour and may decline to undetectable levels following the initial process of tissue collection. As well, there may be a loss of *in vivo* factors regulating the induction of PGHS-2 following the removal of the placenta from the *in utero* environment. Indeed, induction of mRNA for PGHS-2 has been documented by Mitchell *et al.* (1992) who was able to demonstrate the presence of the PGHS-2 mRNA following interleukin-1ß

stimulation of confluent cultured amnion cells. Since increased cytokine levels in gestational tissues are associated with clinical infection, it is unlikely that a similar mode of induction existed in our tissues which were histologically confirmed to be free of infection. However, sub-clinical infections or other situations in which cytokine levels are increased in the absence of infection might possibly have resulted in the expression of PGHS-2.

To date, the mechanism(s) directly responsible for human labour onset have not been clearly defined. The present study examined the changes in PGHS specific activity in the human amnion. We find that increases in PGHS specific activity occur in late gestation immediately prior to the expected time of labour onset and with labour. As well, the observed increases in enzyme activity are not correlated with increased amounts of irPGHS, suggesting that overall changes in the enzyme turnover rate may be of importance in the regulation of PGHS activity. The identification of PGHS-2 in the amnion suggests a role for both cytokine and lipopolysaccaride-mediated increases in PGHS activity occurring at labour both in the presence and absence of clinical infection. We propose that an important step in the initiation and/or maintenance of labour may be an increase in PGHS activity prior to labour onset that facilitates the production of PG required for parturition.

2.5. Figures

Figure 2-1: The effect of term labour onset on human amnion PGHS specific activity. Microsomes were prepared and incubated as described in materials and methods. Term C/S amnion were obtained following elective C/S and post-labour tissue obtained following spontaneous labour onset and delivery. PGHS specific activity, expressed in pg PGE_2/μg protein/minute, was significantly higher ($p<0.05$) after term labour. Data for each group are expressed as the $\bar{x} \pm$ S.E.M. with the sample size and gestational age range of each study group shown on the abscissa.

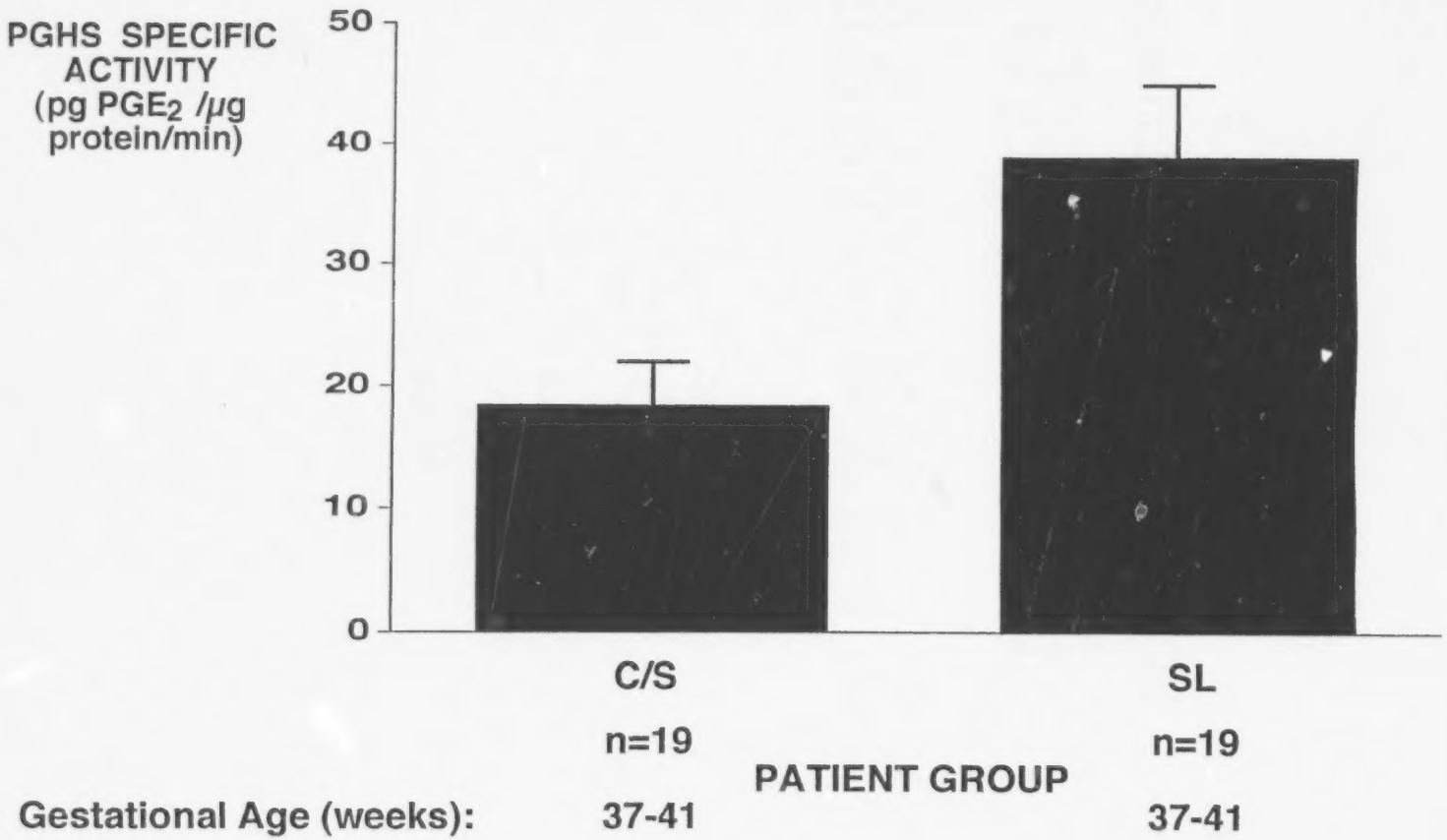

PGHS SPECIFIC ACTIVITY (pg PGE_2 /μg protein/min)
50
40
30
20
10
0
C/S
n=19
SL
n=19
PATIENT GROUP
Gestational Age (weeks):
37-41
37-41

Figure 2-2: The effect of pre-term labour onset on human amnion PGHS specific activity. Microsomes were prepared and incubated as described in materials and methods. Pre-term C/S amnion were collected following obstetrical intervention for either pregnancy induced hypertension, placenta previa or fetal distress or pregnancy termination. Post-labour tissues were collected following spontaneous idiopathic preterm labour and delivery. Data for each group are expressed as the $\bar{x} \pm$ S.E.M. with the sample size and gestational age range of each study group shown on the abscissa. A significant ($p<0.05$) 4-fold increase in PGHS specific activity, expressed in pg PGE_2/μg protein/minute, was observed following pre-term labour onset.

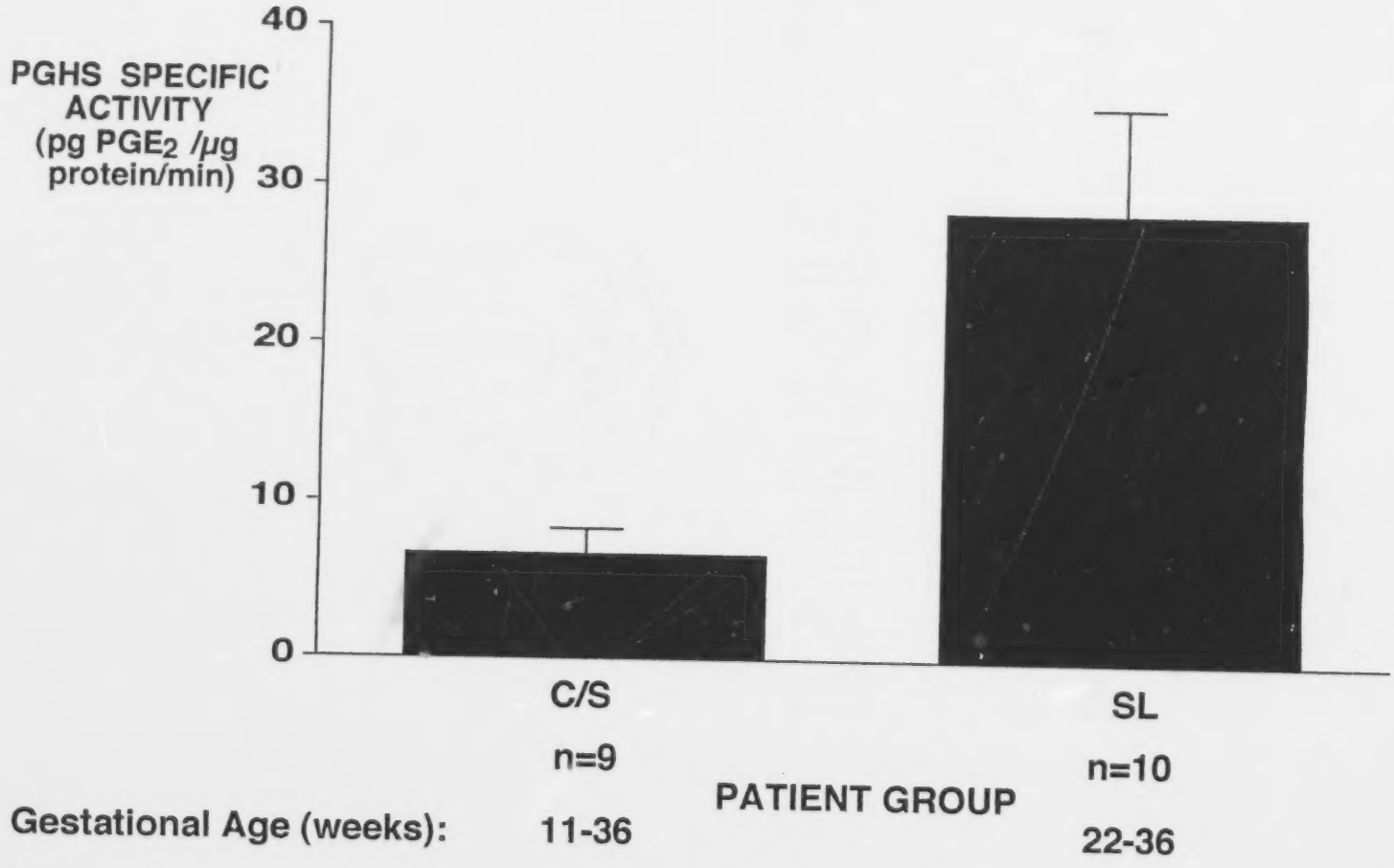

40
30
20
10
0
PGHS SPECIFIC ACTIVITY (pg PGE2 /µg protein/min)
C/S
n=9
SL
n=10
PATIENT GROUP
Gestational Age (weeks):
11-36
22-36

Figure 2-3: Changes in human amnion PGHS specific activity throughout gestation. Term and pre-term C/S tissue were obtained and processed as described with each point representing a single amnion. Calculated PGHS specific activity values were plotted against the gestational age of the tissues at the time of obstetrical intervention as determined by Doppler ultrasound of the fetus. PGHS specific activity (in pg PGE_2/µg protein/minute) remained low in the first and second trimester of pregnancy with a marked significant ($p<0.05$) increase in PGHS specific activity in the final weeks of gestation immediately prior to the expected time of labour onset.

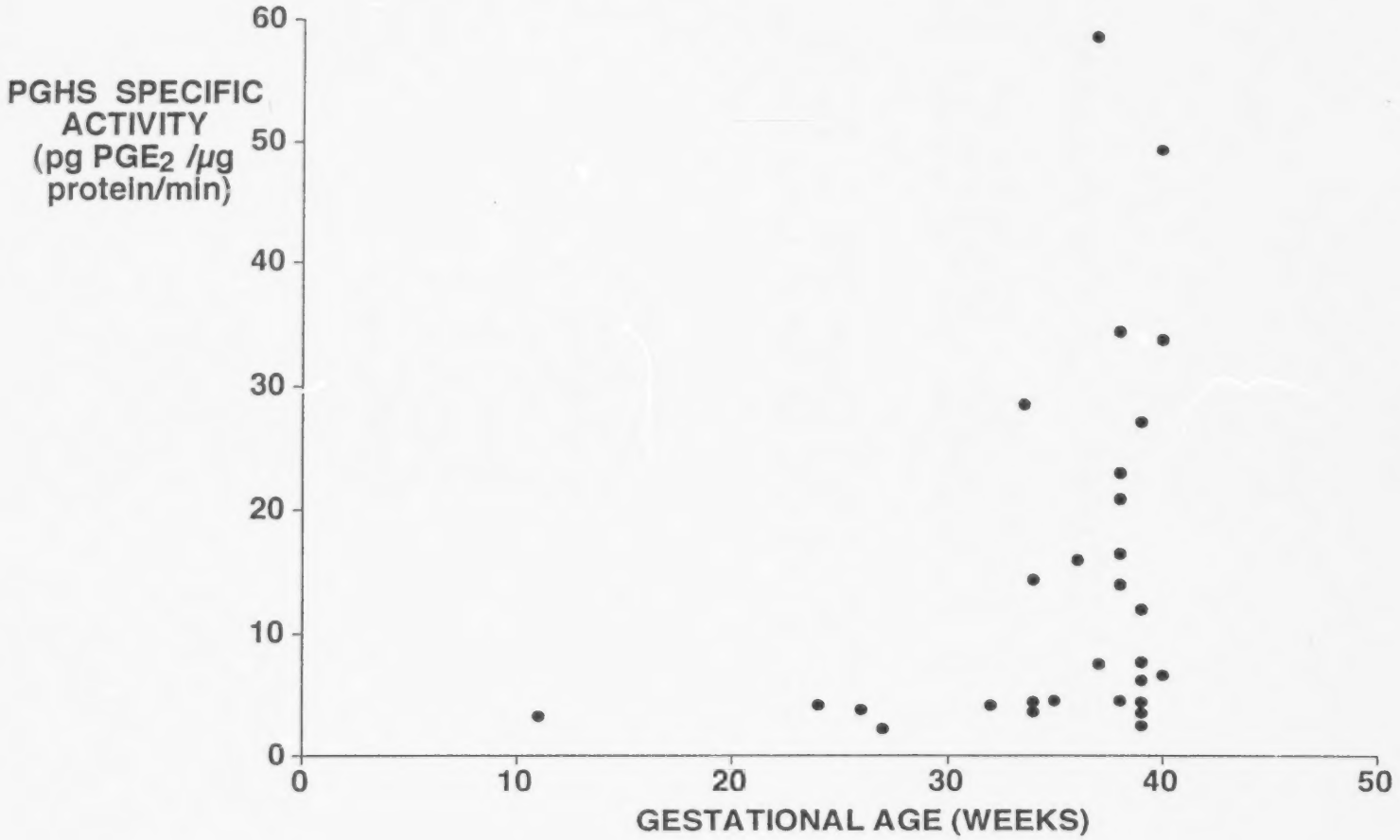

PGHS SPECIFIC ACTIVITY (pg PGE2 /μg protein/min)
60
50
40
30
20
10
0
0
10
20
30
40
50
GESTATIONAL AGE (WEEKS)

Figure 2-4: Immunoblot detection of PGHS. Immunoblotting procedures were performed as described using a rabbit polyclonal Ab raised against ovine PGHS at a final dilution of 1:1000. Bands were visualized using an anti-rabbit IgG alkaline phosphatase conjugate at a final dilution of 1:1000. Minimal signal detection was observed at 0.1 fmol ovine PGHS standard. Lanes 1 and 2 contain ovine PGHS standards at 10 and 1 fmol respectively; lane 3, microsomal amnion fraction from a spontaneous labour amnion (2 μg); lane 4, amnion microsomal fraction from a C/S tissue (2 μg); lane 5, amnion microsomal fraction from a C/S tissue (2 μg); lane 6, amnion microsomal fraction from an SL tissue (2 μg); lane 7, amnion microsomal fraction from a C/S tissue (2 μg); and lane 8, amnion microsomal fraction from an SL tissue (2 μg). There was no difference in the intensity of staining for PGHS as determined by densitometric analysis. Arrows indicate the positions of molecular weight markers.

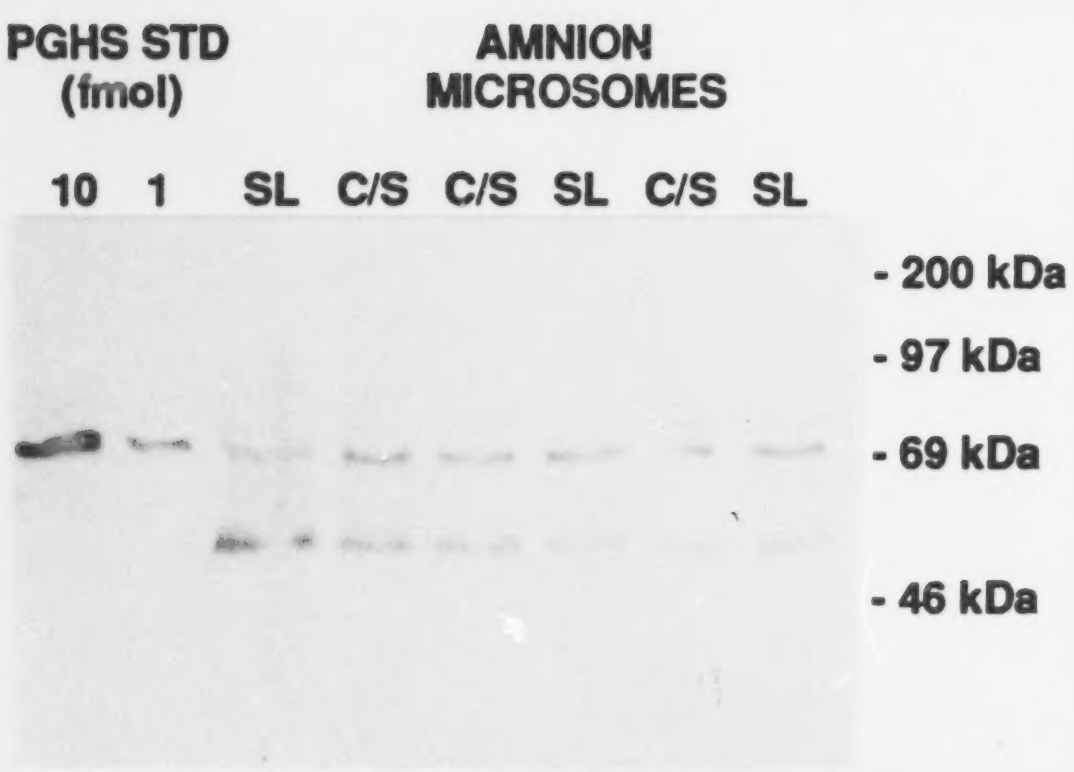

PGHS STD
(fmol)
AMNION
MICROSOMES
10
1
SL
C/S
C/S
SL
C/S
SL
- 200 kDa
- 97 kDa
- 69 kDa
- 46 kDa

Figure 2-5: Correlation between immunoreactive PGHS levels and PGHS specific activity. Each amnion sample used in enzyme kinetic analysis was subjected to immunoblotting procedures using a rabbit polyclonal Ab raised against ovine PGHS, at a final dilution of 1:1000. Bands were visualized using an anti-rabbit IgG alkaline phosphatase conjugate at a final dilution of 1:1000. Each sample was processed using 2 μg of microsomal protein. Immunoreactive bands co-migrating with the authentic ovine standards were analysed with a densitometer to obtain semi-quantitative results. Linear regression analysis indicated that there was no correlation between the amount of irPGHS and calculated PGHS specific activity values ($p>0.05$).

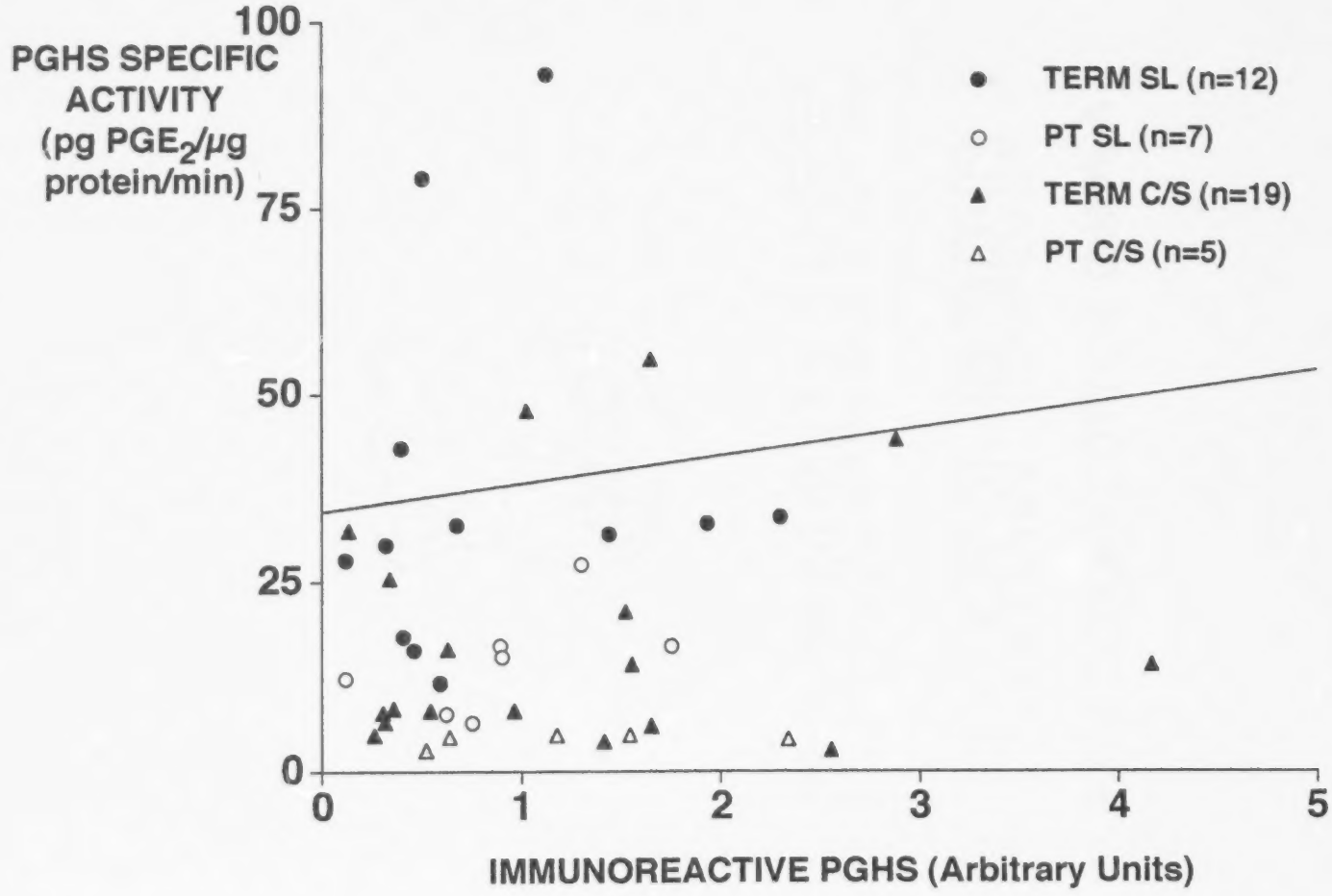

PGHS SPECIFIC ACTIVITY (pg PGE2/μg protein/min)
100
75
50
25
0
0
1
2
3
4
5
IMMUNOREACTIVE PGHS (Arbitrary Units)
TERM SL (n=12)
PT SL (n=7)
TERM C/S (n=19)
PT C/S (n=5)

Figure 2-6: A) Immunodetection of PGHS-2 in human amnion microsomal samples. Decreasing amounts of PGHS-2 standard and one amnion microsomal preparation were run and incubated with a rabbit polyclonal Ab raised against ovine PGHS at a final dilution of 1:1000. From left to right, lane 1: PGHS-2 standard, 800 fmol; lane 2: PGHS-2 standard, 200 fmol; lane 3: PGHS-2 standard, 100 fmol; lane 4: PGHS-2 standard, 50 fmol; lane 5: amnion microsomes 40 μg; lane 6: amnion microsomes, 20 μg; lane 7: amnion microsomes, 10 μg; lane 8: amnion microsomes 5 μg. Bands were visualized using an anti-rabbit IgG horseradish peroxidase conjugate at a final dilution of 1:2000. Maximal signal detection under minimal background conditions was observed at between 10 and 20 μg protein. Subsequent analysis of microsomes for PGHS-2 detection was performed at 14 μg microsomal protein. Molecular weight markers are shown to the right of the blot. B) Immunoblot detection of PGHS-2 using pre-immune serum. Blots were prepared as above with 200 fmol sheep PGHS-2 standard and incubated in the presence of pre-immune serum at a final concentration of 1:1000. Band visualization was attempted by using an anti-rabbit IgG horseradish peroxidase conjugate at a final dilution of 1:2000. No banding pattern was observed with the addition of the pre-immune serum.

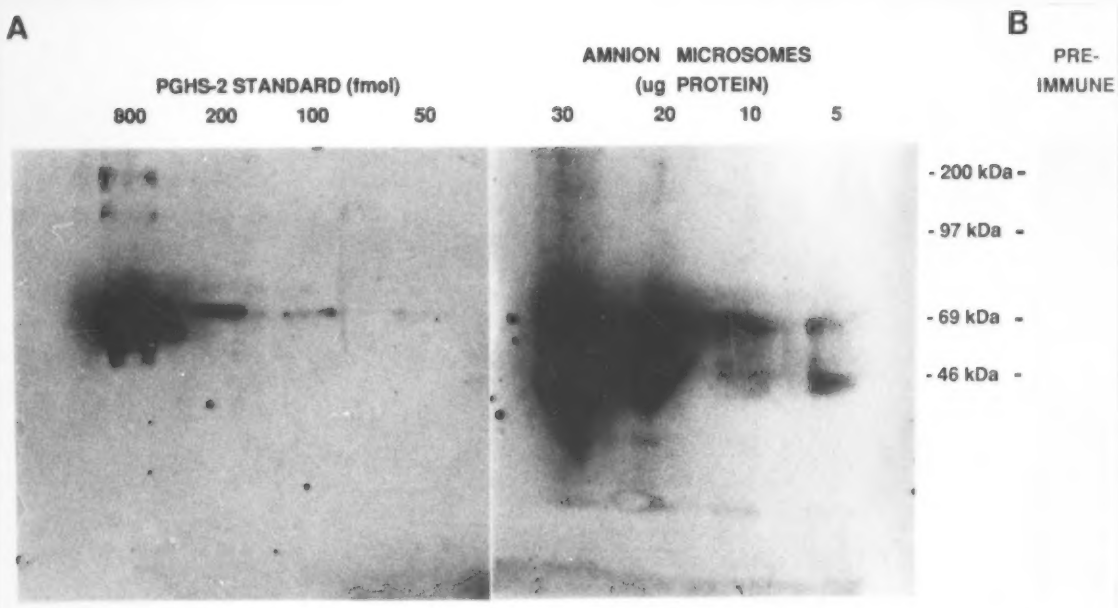

A
PGHS-2 STANDARD (fmol)
800
200
100
50
AMNION MICROSOMES
(ug PROTEIN)
30
20
10
5
B
PRE-
IMMUNE
- 200 kDa -
- 97 kDa -
- 69 kDa -
- 46 kDa -

Figure 2-7: Correlation between immunoreactive PGHS-2 levels and PGHS specific activity. Amnion samples used in enzyme kinetic analyses were subjected to immunoblotting procedures using a rabbit polyclonal Ab raised against a unique 17-amino acid sequence on PGHS-2 at a final dilution of 1:1000. Bands were visualized using an anti-rabbit IgG horseradish peroxidase conjugate at a final dilution of 1:2000. Each sample was processed using 14 µg of microsomal protein. Immunoreactive bands co-migrating with the authentic ovine standards were analysed with a densitometer to obtain semi-quantitative results. Linear regression analysis indicated that there was no correlation between the amount of irPGHS and calculated PGHS specific activity values ($p>0.05$).

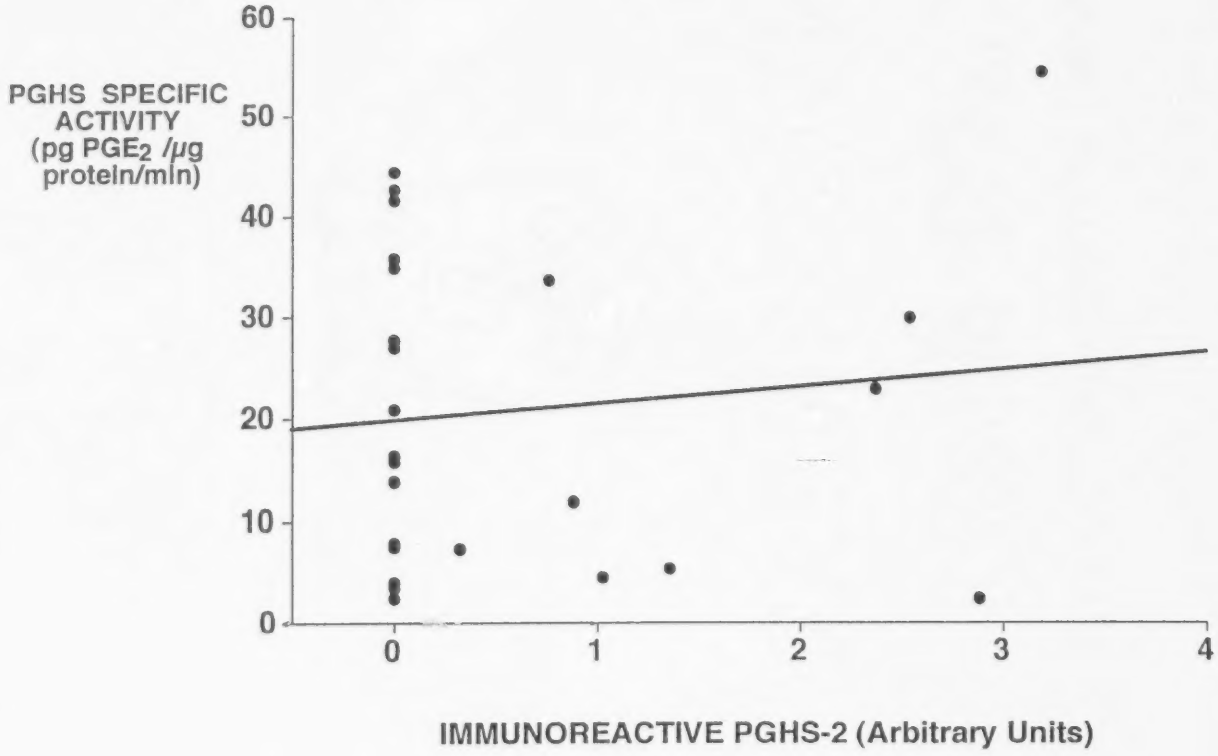
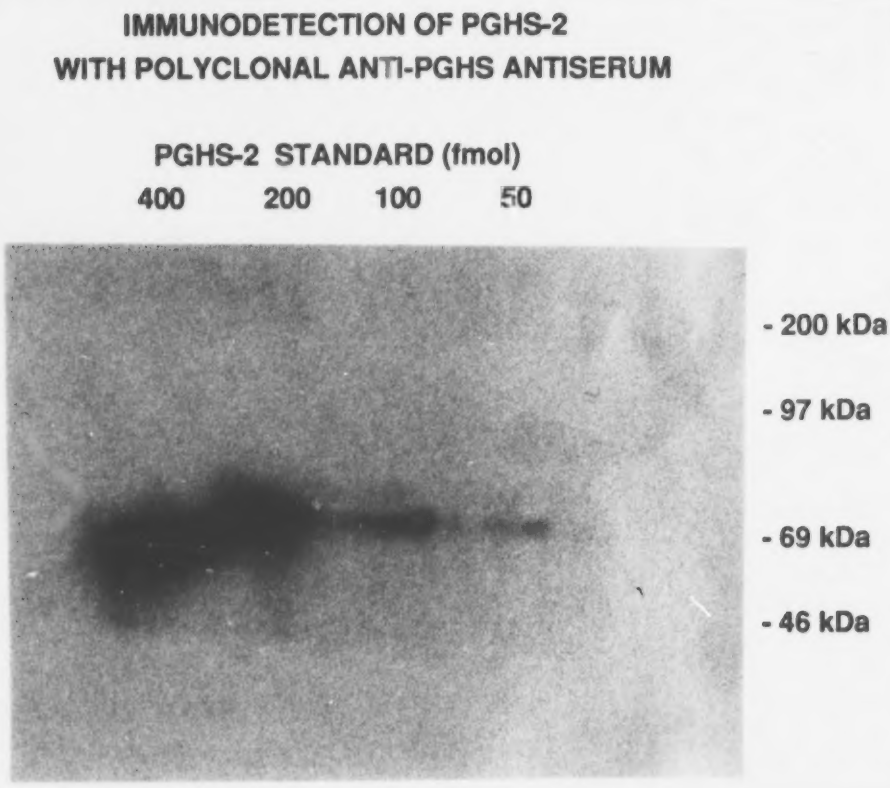

PGHS SPECIFIC ACTIVITY (pg PGE_2 /μg protein/min)
60
50
40
30
20
10
0
0
1
2
3
4
IMMUNOREACTIVE PGHS-2 (Arbitrary Units)

Figure 2-8: Immunodetection of PGHS-2 with polyclonal anti-PGHS antiserum. Immunoblots were prepared as described. Decreasing amounts of PGHS-2 standard were run and incubated with a rabbit polyclonal Ab raised against ovine PGHS at a final dilution of 1:1000. Bands were visualized using an anti-rabbit IgG horseradish peroxidase conjugate at a final dilution of 1:2000. Minimal signal detection was observed at between 50 and 100 fmol of recombinant PGHS-2 standard. Molecular weight markers are shown to the right of the blot.

IMMUNODETECTION OF PGHS-2
WITH POLYCLONAL ANTI-PGHS ANTISERUM



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3. Regulation of PGHS *in vitro*

3.1. Introduction

Prostanoids synthesized by the intrauterine tissues play important roles in the initiation and/or maintenance of labour in a number of species (Challis and Olson, 1988). In pregnant women, the amnion membrane is a major intrauterine source of prostaglandins (Mitchell, 1986). The amnion produces increased amounts of prostaglandin E_2 (PGE_2) at labour (Mitchell, 1986; Casey and MacDonald, 1986), which suggests that the stimulation of amniotic PGE_2 synthesis is an essential part of the physiological process leading to birth.

Prostaglandin endoperoxide H synthase (PGHS[1], EC 1.14.99.1) catalyses the first, committed step of prostaglandin synthesis from arachidonic acid (Smith *et al.*, 1991). PGHS activity increases in the amnion at parturition (Okazaki *et al.*, 1981; Olson *et al.*, 1991), and the findings of Gaffney *et al.* (Gaffney *et al.*, 1990) indicate that the rate of PGHS synthesis in the chorioamnion is higher following labour than before the spontaneous onset of labour. Therefore, the increase of amnion PGE_2 production at term labour may be the consequence, at least in part, of stimulated synthesis and high concentration of PGHS in this tissue.

PGHS activity is controlled in many cells by the regulation of enzyme mass. As reviewed recently by DeWitt (DeWitt, 1991) and Smith *et al* (Smith *et al.*, 1991), the expression of PGHS is affected by a variety of agonists including growth factors, cytokines, steroids, and tumour promoters in fibroblasts, vascular cells, monocyte- and macrophage-like cells, ovarian, uterine, and renal cells. Confluent cultured amnion cells also respond to a number of effectors with increased PGE_2 output (see Olson *et al.*, 1990 for review). Several of these, such as epidermal growth factor, interleukin 1 (IL-1), dexamethasone and the

tumour promoter phorbol esters were demonstrated to stimulate the *de novo* synthesis of PGHS (Smith *et al.*, 1991; DeWitt, 1991; Olson *et al.*, 1990). Recent data indicate, however, that fresh amnion tissue or freshly dispersed amnion cells respond differently to certain agonists compared to confluent cultured amnion cells. Contrary to their effect on confluent cell cultures, glucocorticoids inhibit the prostaglandin production in fresh amnion (Gibb and Lavoie, 1990), and epidermal growth factor has no effect on the PGE_2 output of freshly dispersed amnion cells (Gibb and Lavoie, 1990). Since the responses of freshly isolated amnion may reflect the *in vivo* responsiveness of amnion tissue more closely than the responses of confluent cultured amnion cells, we have developed a tissue incubation system to study the mechanisms regulating amniotic prostaglandin production. Using this model, we have shown that the activation of protein kinase C (PKC) by phorbol esters increases the PGE_2 production of the amnion (Zakar and Olson, 1992).

In the present experiments, we determined the effects of glucocorticoids and phorbol esters on the synthesis rate, enzyme activity, and immunoreactive protein concentrations of PGHS in amnion tissue. We hypothesized that glucocorticoids inhibit and PKC activators stimulate amniotic prostaglandin production by affecting PGHS enzyme activity levels in the amnion membranes. By demonstrating that amniotic PGHS may be positively and negatively regulated we strongly suggest that one of the factors which controls intrauterine prostaglandin synthesis at term is the activity of PGHS in the fetal membranes.

3.2. Materials and Methods

Materials

[5,6,8,11,12,14,15-N-^{3}H]PGE_2 (sp. act., 140 Ci mmol^{-1}) was obtained from Amersham Canada, (Oakville, ON.). The following materials were purchased from Sigma Chemical Co. (St. Louis, MO.): Phorbol esters, 4ß-phorbol, calf thymus DNA, bis-benzimidazole (Hoechst 33258), fatty acid free bovine serum albumin, cycloheximide, actinomycin D, acetylsalicylic acid, indomethacin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), diethyldithiocarbamic acid (DDC), and all steroids except for RU486 (17ß-hydroxy-11ß-(4-dimethylaminophenol)-17α-(prop-1-ynyl)estra-4,9-diene-3-one), which was the product of Roussel UCLAF (Romainville, France). Prostaglandin E_2, prostaglandin endoperoxide H synthase (PGHS) purified from sheep seminal vesicles, and rabbit polyclonal antibody raised against sheep seminal vesicle PGHS were from Cayman Chemical (Ann Arbor, MI.). Arachidonic acid and reduced glutathione (GSH) were bought from Nu-Chek Preparations (Elysian, MN.) and Boehringer Mannheim Canada (Laval, P. Q.), respectively. The Sep-Pak C_{18} cartridges were the products of Waters (Milford, MA.). Anti-rabbit IgG (Fc)-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), and nitro blue tetrazolium (NBT) were supplied by Promega Corp. (Madison, WI.). Reagents for SDS-polyacrylamide gel electrophoresis and for the electrophoretic transfer of proteins from polyacrylamide gels to membranes (MagnaGraph Nylon, MSI, Westboro, MA.) were from Fisher Scientific (Ottawa, ON). Prestained SDS-polyacryamide gel electrophoresis standards were obtained from Bio-Rad Canada (Mississauga, ON.). All other chemicals were of analytical (ACS) purity.

Methods

Tissues. Reflected amnion membranes were isolated from placentas delivered spontaneously at term after uncomplicated pregnancies. The patients received no drugs during labour other than analgesics. The pregnancies were singleton fetuses with normal outcomes. The use of these tissues was approved by the University of Alberta Ethics Review Committee.

Preparation and incubation of minced amnion tissue. The procedures used have been described in detail by Zakar and Olson (1992). Briefly, the amnion membranes were placed in synthetic ("pseudo-") amniotic fluid (PsAF, for composition and properties see refs. Zakar and Olson, 1992 and Schwartz *et al.,* 1977), cleaned, minced, washed 3 times, and preincubated in PsAF for 3 h with one change of medium at 1.5 h. Fresh medium with agonists, dissolved in dimethyl sulfoxide or ethanol, was then added for 14-16 h at 37°C. The concentration of the solvents was less than 0.05% in the incubation media, which had no effect on the PGE_2 production of the tissues. After the agonist treatments, the tissue samples (each representing 1-5 μg of DNA) were incubated for 2 h in 1 ml of fresh PsAF supplemented with 10 μmol arachidonic acid l^{-1}, and PGE_2 output was determined by RIA.

In experiments involving acetylsalicylic acid, (ASA), treatment, the drug was added to the tissues at 0.4 mM final concentration for the second 1.5 h of the preincubation period. The tissues were then washed twice with PsAF, and incubated with agonists for 16 h at 37°C. Fresh medium with 10 μM arachidonic acid was then added for 1 h, and PGE_2 output was determined. The efficiency of the ASA treatment protocol to inhibit PGE_2 synthesis was determined in control incubations, where the tissues were treated with ASA in PsAF for 30 min, followed by fresh medium with ASA and arachidonate for 1 h. PGE_2 output

during the arachidonate plus ASA treatment period was determined, and compared with the PGE_2 output of tissue treated with arachidonate but not with ASA.

To determine the steady state values of PGHS enzyme activity and PGHS immunoreactive protein, the amnion membranes were cut into 1-4 cm^2 pieces, and 3-7 g of tissue (wet weight) was incubated in 50 ml of medium with agonists. An aliquot of the incubation medium was saved for PGE_2 determination, and the tissue pieces were blotted on filter paper (Whatman 3M), rinsed with PsAF, blotted again, and frozen in liquid N_2. The frozen tissues were stored at -74°C until further processing.

PGE_2 determination. PGE_2 content was determined directly from the incubation media with a specific radioimmunoassay (Evans *et al.,* 1981) characterized previously by Olson *et al.* (1984). The intra assay and inter assay coefficients of variation of this procedure are 6.0 % and 6.9 %, respectively (at 2000 pg ml^{-1} PGE_2 concentration, calculated using the recovery equation $y=1.10x-30$; $r=0.993$; $p<0.001$; Student's t test) (Olson *et al.,* 1983). Blanks containing incubation media with arachidonic acid were included in each assay, and experiments with detectable background levels were excluded from the study.

DNA extraction and determination. DNA was extracted from the tissues, and determined by a fluorimetric method using procedures of Zakar and Olson (1992). In brief, the samples were homogenized in ice cold 1mol NH_4OH l^{-1}; 10 mmol EDTA l^{-1}, and incubated over 5 mol H_2SO_4 l^{-1} at room temperature overnight. An aliquot of the supernatant was used to measure DNA content with a *bis*--benzimidazole-fluorescence enhancement assay (Downs and Wilfinger, 1983). Calf thymus DNA was used as standard.

Determination of PGHS activity. The method has been described

(Smieja *et al.*, 1993), and was used with minor modifications. The frozen tissue samples were pulverized in liquid N_2, and homogenized in a solution containing 0.25 mol sucrose l^{-1}; 50 mmol Tris-HCl l^{-1} (pH 8.0); 2.5 mmol tryptophan l^{-1}; 2 mmol EDTA l^{-1}; 1 mmol DDC l^{-1}; 1 mmol PMSF l^{-1}; 10 μg ml^{-1} leupeptin. The homogenate was centrifuged at 2000 g at 2°C for 10 min, and the floating layer of fat was removed. The aqueous supernatant was ultracentrifuged with 105 000 g at 2°C for 60 min, and the particulate fraction, homogenized in 50 mmol Tris-HCl l^{-1} (pH 8.0); 2.5 mmol tryptophan l^{-1}; 2 mmol EDTA l^{-1}; 1 mmol DDC l^{-1}, was used as enzyme preparation.

The PGHS enzyme reaction mixtures contained 50 mmol Tris-HCl l^{-1} (pH 8.0); 2 mmol EDTA l^{-1}; 4 mmol tryptophan l^{-1}; 1 mmol GSH l^{-1}; 10 μmol arachidonic acid l^{-1}; and 50 μl of enzyme preparation (3 - 25 μg protein) in a final volume of 250 μl. The cofactor and the arachidonate concentrations were shown previously to be optimal and saturating, respectively (Smieja *et al.*, 1993). Reactions were started with enzyme, and allowed to proceed at 37°C for 4 min, representing initial velocity. Reactions were stopped by adding 2.5 ml of 50 mmol sodium citrate l^{-1} (pH 3) containing 15 % (v/v) ethanol, and prostaglandins were extracted using Sep-Pak C_{18} cartridges as described (Smieja *et al.*, 1993). PGE_2, recovered with 85-95 % efficiency, was determined in the extracts with RIA. Each enzyme preparation was assayed in triplicate at two protein concentrations. Results were normalized to protein content, and expressed as ng PGE_2 produced/μg protein/ 4 min.

SDS-polyacryamide gel electrophoresis, membrane transfer, and immunodetection of PGHS. Low speed (2000 x g, 10 min) supernatants of the tissue homogenates were subjected to ultracentrifugation (105,000 x g, 60 min). The pellets were dissolved in electrophoresis sample buffer (Laemmli, 1970) by heating at 90°C for 10 min. The proteins (2 -4 μg/lane) were

separated by electrophoresis in 7.5 % SDS-polyacrylamide gels according to Laemmli (1970), and transferred electrophoretically (100 V, 60 min at 4°C) to MagnaGraph Nylon membranes using the method described by Towbin et al. (Towbin *et al.*, 1979). The membranes were blocked by incubating in 5 % non fat dry milk dissolved in TBS (25 mmol Tris-HCl l^{-1}, pH 7.5; 0.5 mol NaCl l^{-1}) at room temperature for 1 h. Rabbit polyclonal anti-PGHS antiserum, diluted 1: 1000 in TBS, 5% nonfat dry milk, 0.02 % sodium-azide, was then added to the membranes for 14 h at room temperature. The primary antibody was removed by three 20 min washings with TBS, 0.3 % Tween 20 , and the membranes were incubated for 2 h with an alkaline phosphatase conjugated anti-rabbit IgG antibody, diluted 10,000 fold with TBS, 0.3% Tween 20. After three 20 min washes with TBS, 0.3 % Tween 20, alkaline phosphatase buffer (100 mmol NaCl l^{-1}; 50 mmol Tris - HCl l^{-1}, pH 10; 2 mmol $MgCl_2$ l^{-1}) containing a chromogenic alkaline phosphatase detection system (BCIP/NBT) was added to the membranes. The color was developed at room temperature for 15 - 30 min. The blots were scanned with an LKB Ultroscan laser densitometer, and the peaks corresponding to immunoreactive PGHS were integrated manually.

Protein determination. Proteins were solubilized by incubating the particulate fractions in 0.2 mol NaOH l^{-1} at 37°C overnight. The NaOH was then neutralized with an equivalent amount of HCl, and the protein concentrations were determined according to Bradford's dye binding method as described (Zakar and Olson, 1988). Bovine serum albumin was used as standard.

Statistical analysis and presentation of data. PGE_2 values on all figures were normalized individually to the DNA content of the corresponding tissue sample. The data were then subjected to analysis of variance using a computer program (SuperANOVA, Abacus Concepts, Inc., Berkley, CA), and where a significant F value was obtained, treatment means were separated using the

Newman-Keuls post hoc test. All figures except Figure 3-2 display individual experiments where each point or bar represents the mean of 4 parallel incubations ± SEM. Figure 3-2 shows data obtained with 3 to 6 patients. $P<0.05$ was considered significant. Further details of data analysis are presented in the legends to the figures. PGHS enzyme activity values were analysed by analysis of variance in which separate error terms were identifed for experimental differences, patient to patient differences, and treatment differences. The data for the grouped means and the statistical analysis for these data are presented in appendix 4. This analysis showed no significant effect of cortisol and TPA on PGHS enzyme activity.

3.3. Results

We investigated the possibility that a decrease of PGHS activity was involved in the inhibition of amniotic PGE_2 production by glucocorticoids (Gibb and Lavoie, 1990) (Fig.3-1). Cortisol inhibited the arachidonate stimulated PGE_2 output with an IC_{50} of approximately 10^{-9} mol l^{-1}. A parallel set of tissue samples was incubated with cortisol in the presence of10^{-7} mol l^{-1} RU486 , a glucocorticoid receptor antagonist (Jung-Testas and Baulieu, 1983), in order to test the involvement of the glucocorticoid hormone receptor in the action of the steroid. Unexpectedly, RU486 also blocked PGE_2 output, with a tendency to antagonize the effect of higher concentrations (10^{-7}-10^{-6} mol l^{-1}) of cortisol.

Cortisol, RU486, and dexamethasone, added at 10^{-7} mol l^{-1} concentration, caused a significant ($p<0.05$) decrease of arachidonate stimulated prostaglandin output, while 10^{-7} mol l^{-1} estradiol, progesterone, dehydroepiandrosterone sulfate, testosterone, and 17-hydroxyprogesterone did not influence PGE_2 production relative to the vehicle treated controls (Fig. 3-2). Thus, the inhibitory effect involved a decrease in PGHS activity, and was specific to steroids that bind to the glucocorticoid receptor.

Cycloheximide (100 µg ml^{-1}), a protein synthesis inhibitor, blocked PGE_2 synthesis by more than 90% (Fig.3-2). Amino acid incorporation into acid precipitated material was blocked by this concentration of cycloheximide to a similar extent (>95%, data not shown), confirming previous observations that protein synthesis was required for prostaglandin production by the amnion (Gibb and Lavoie, 1990; Zakar and Olson, 1992), and suggesting that the maintenance of PGHS activity levels is dependent on continuous protein synthesis.

Figure 3-3 shows the effect of TPA, a PKC activating phorbol ester (Castagna *et al.,* 1982) and strong stimulant of amnion PGE_2 production (Zakar

and Olson, 1992), on the arachidonate promoted PGE_2 output. TPA increased the conversion of arachidonic acid to PGE_2 in a concentration dependent fashion. Maximal stimulation was reached at 10^{-7} mol TPA l^{-1}; half maximal increase was achieved with approximately 15 nmol l^{-1} concentration of the phorbol ester. When the tissues were co-incubated with TPA and 10^{-6} mol cortisol l^{-1}, the basal as well as the stimulated prostaglandin production was reduced by 40-60% without affecting the EC_{50} of the phorbol ester.

Treatment with TPA or phorbol-12,13-dibutyrate (PBD), which are strong activators of PKC (Castagna *et al.*, 1982), significantly ($p<0.05$) increased the prostaglandin production during subsequent stimulation with arachidonic acid (Fig.3-4). Moreover, their efficacy (TPA>PBD) corresponded to their relative potency to activate PKC. 4-methyl TPA or 4ß-phorbol, which are weak tumour promoters and do not activate PKC, were ineffective.

Since PGHS irreversibly self-inactivates while performing its catalytic function (Smith and Lands, 1972), the level of PGHS activity most likely reflects the rate of *de novo* enzyme synthesis. We studied the effect of TPA and cortisol on the *de novo* synthesis of amniotic PGHS using tissues pretreated with acetylsalicylic acid (ASA) (Fig.5). ASA irreversibly inactivates PGHS (Smith and Marnett, 1991), and prostaglandin production after ASA pretreatment requires the synthesis of new PGHS protein. Control incubations, by adding arachidonate for the last 60 min of the ASA treatment interval, showed that ASA inhibited PGE_2 output by approximately 90 % (compare the two bars on the left side of Fig.3-5). However, the ability of the tissue to produce PGE_2 from arachidonate returned to the pre-treatment value almost completely after 16 h. TPA (10^{-7} mol l^{-1}) significantly increased, and cortisol (10^{-7} mol l^{-1}) significantly decreased ($p<0.05$) the recovery as compared to tissue incubated without agonists. Combined treatment with TPA and cortisol resulted in significantly

lower recovery than with TPA alone, but in higher recovery than in the absence of agonists. Furthermore, actinomycin D (2 μg ml^{-1}), an RNA synthesis inhibitor, blocked the phorbol ester stimulation as well as the glucocorticoid inhibition of PGHS synthesis, but did not affect significantly the spontaneous recovery of the enzyme. These results indicate that the phorbol ester stimulated and the glucocorticoid inhibited the *de novo* synthesis of PGHS in an RNA synthesis dependent manner. At the same time, the spontaneous recovery of PGHS did not require continuous RNA synthesis.

Alterations of the PGHS synthesis rate may lead to changes in the steady state level of the enzyme activity. To test this possibility, and to obtain direct evidence that glucocorticoids and PKC activators influence the amount of PGHS in the amnion, we have determined the effects of cortisol and TPA on the specific activity of PGHS in amnion microsomes. The PGHS specific activity of the microsomal preparations from untreated tissues was 40.08 ± 14.88 ng PGE_2 mg^{-1} protein per 4 min (mean±SEM, n=6 patients). Cortisol decreased the level of PGHS activity to 36.81±11.88 ng PGE_2 mg^{-1} protein per 4 min ($p<0.05$; ANOVA, cf. appendix 4). Enzyme activity in microsomes from TPA-treated tissues was 39.92±8.07 ng PGE_2 mg^{-1} protein per 4 min, not different from the control. However, using logarithmically transformed data, a significant stimulation by TPA was revealed (control: 3.17, ± 0.48, ; TPA: 3.52 ± 0.27 ; mean, ± SEM, ; $p<0.05$, ANOVA, cf. appendix 4). Although these results agreed in general with the results of experiments in which the arachidonic acid promoted PGE_2 output was measured, they also suggested a complex relationship between PGHS synthesis rates and steady state levels of PGHS activity.

Changes in the synthesis rate and steady state activity levels of PGHS may accompany corresponding changes in the amount of enzyme protein. To

determine the effects of TPA and cortisol on the level of PGHS mass in the amnion, we measured the amount of immunoreactive PGHS in amnion membranes. Figure 3-6 shows representative PGHS immunoblots obtained with tissues from two patients, and Table 1 contains the results of the densitometric quantification of PGHS bands. PGE_2 output during the agonist treatment periods was also determined, and presented in Table 1. In amnion no.1(Figure 3-6), a single immunoreactive band co-migrating with the ovine PGHS standard (70 kDa) was detected (control, lane 2). This band disappeared when the blots were exposed to the PGHS antiserum in the presence of excess PGHS verifying the specificity of the immunodetection (not shown). The amount of PGHS in this preparation was between 0.5 - 5 fmol/µg protein, as judged from the intensity of bands generated by known amounts of purified PGHS on the same blot (lanes 1 and 8; Fig 3-6, Table 3-1). TPA treatment increased, and cortisol decreased the immunoreactive PGHS content of the particulate fraction (lanes 3 and 5, respectively). Moreover, the phorbol ester caused the expansion of the band identified as PGHS, which indicates the formation of immunoreactive material with a molecular weight higher than that of the standard. In agreement with the changes of enzyme protein levels, the PGE_2 output of the tissue increased over 8 fold in the presence of the phorbol ester, and decreased by more than 50 % during cortisol treatment (Table 3-1).

Also, tissue samples from the same amnion were treated with agonists in the presence of indomethacin. Indomethacin inhibits cyclooxygenase activity, and stabilizes the enzyme against proteolytic degradation (Mizuno *et al.*, 1982; Kulmacz, 1989). Accordingly, in amnion no.1, indomethacin increased the level of immunoreactive PGHS in the absence as well as in the presence of cortisol or TPA (Fig. 3-6, Table 3-1). The maximal increase was seen with 5 µmol indomethacin l^{-1}, while 1 µmol or 10 µmol indomethacin l^{-1} had little or no effect

(not shown). Furthermore, cortisol inhibited, and TPA stimulated PGHS protein accumulation in indomethacin treated tissues; and the effect of the phorbol ester was particularly enhanced by the enzyme inhibitor (lanes 5 and 4). The PGE_2 output of the tissues was blocked by more than 85 % with 5 μmol indomethacin l^{-1} under all experimental conditions (Table 3-1). Responses such as those of amnion no.1 were seen with 3 of the 5 amnion membranes included in the study.

Amnion no.2, a representative of 2 of the 5 tissues studied, exhibited another type of response to the above treatments (Fig.3-6, lower panel). This tissue contained more PGHS than amnion no.1 (>10 fmol μg^{-1} protein) in the particulate fraction, and the PGHS band (lane 2) was wider than the band generated by the PGHS standard. This suggests an increased heterogeneity of the PGHS protein in this amnion as compared to the ovine enzyme, including the possible presence of a protein doublet. The intensity of the PGHS band decreased after incubating the tissue with cortisol as well as with TPA, and a single band co-migrating with the ovine PGHS appeared (lanes 5 and 3, respectively). The PGE_2 output of amnion no.2 was greater than that of amnion no.1, which was in agreement with the relative levels of immunoreactive PGHS(Table 3-1). Cortisol treatment decreased the prostaglandin production of amnion no.2 by 66 %, but TPA caused a more than twofold increase of PGE_2 output despite its inhibitory effect on PGHS protein accumulation. Indomethacin caused an increase in PGHS protein levels in this tissue only in the presence of TPA (compare lanes 3 and 4), and did not influence immunoreactive enzyme levels in the cortisol treated tissue. After incubation with indomethacin alone, a single PGHS band co-migrating with the ovine standard was detected in amnion no.2 (lane 7). The amount of immunoreactive material in this band, determined by densitometry, was slightly diminished as compared to the control

(lane 2).

In summary, cortisol consistently decreased the amount of immunoreactive PGHS and the production of PGE_2 by the amnion tissues. Phorbol ester stimulated the PGE_2 output of all tissues studied, which was accompanied by an increase of PGHS protein content in some tissues, and a decrease in immunoreactive PGHS levels in others. Indomethacin consistently increased the amount of PGHS protein only in TPA-treated tissues.

3.4. Discussion

The rate limiting step of the conversion of arachidonate to prostaglandins is the oxygenation of arachidonic acid to prostaglandin endoperoxides by PGHS (Needleman *et al.*, 1986; Smith *et al.*, 1991). In agreement with this, the incubation of amnion tissue with 10 µmol arachidonic acid l^{-1}, a concentration which saturates amniotic PGHS in a cell free assay system (Smieja *et al.*, 1993), increases PGE_2 output maximally (López Bernal *et al.*, 1988). The inclusion of 10 µmol arachidonate l^{-1} in the medium of our tissue mince incubation system also caused a significant stimulation of PGE_2 production (0.437 ± 0.053 versus 0.662 ± 0.039 ng μg^{-1}DNA per 2h; mean ± SEM, n=8 patients, $p<0.01$, Student's t-test). Therefore, we assessed PGHS activity in the amnion by determining the capacity of the tissue to convert arachidonate to PGE_2.

In many tissues and cells, glucocorticoids inhibit prostaglandin production (Smith *et al.*, 1991; Smith and Marnett, 1991; Russo-Marie, 1990). However, the intracellular mechanisms mediating this physiologically important action of corticosteroids are unclear. In the present investigation, we provided evidence showing that glucocorticoids diminish amniotic PGE_2 production by inhibiting the activity of PGHS, the enzyme catalyzing the rate limiting step of prostaglandin synthesis from arachidonic acid. We demonstrated that cortisol, dexamethasone, and RU486, which bind to the glucocorticoid receptor, specifically inhibited the production of PGE_2 from exogenous arachidonate. Furthermore, cortisol treatment resulted in a decrease in the levels of PGHS activity and immunoreactive PGHS protein in the particulate (microsomal) fraction. The recovery of arachidonate-stimulated PGE_2 synthesis after irreversible inactivation of PGHS by ASA treatment was also reduced by

cortisol, suggesting that the steroid inhibited the synthesis of new enzyme protein. Interestingly, RU486, an established glucocorticoid and progesterone receptor antagonist, mimicked the effect of the glucocorticoids on arachidonate promoted prostaglandin output. Agonistic effects of this steroid were reported in the human pituitary (Laue *et al.,* 1988) and endometrium (Gravanis *et al.,* 1985), and in monkey endometrium *in vivo,* (Wolf *et al.,* 1989) and in human peripheral blood mononuclear cells *in vitro* (Van Voorhis *et al.,* 1989). Clearly, the human amnion is another tissue where RU486 may act as a steroid hormone agonist.

Experiments with protein and RNA synthesis inhibitors provided information about the mechanism of the glucocorticoid action. Inhibition of protein synthesis with cycloheximide blocked the arachidonate stimulated PGE_2 production by 90%, indicating that continuous protein synthesis was required for the conversion of arachidonic acid to PGE_2. PGHS itself may be one of the proteins which are continuously replenished, since it self-inactivates while performing its normal catalytic function, and has to be synthesized continuously to maintain a steady state level of activity. In agreement with this, the data in Fig. 5 show that PGHS activity in amnion tissue recovered virtually completely during 16 h following ASA pretreatment. The recovery was independent of RNA synthesis, since it was not inhibited by actinomycin D. This suggests that pre-existing RNA molecules were utilized for the synthesis of new PGHS. Cortisol inhibited this process in an RNA synthesis dependent fashion. On the basis of these observations it is reasonable to suggest that the glucocorticoid inhibited the synthesis of PGHS at a post-transcriptional step by inducing a regulatory protein (and/or RNA species). Post-transcriptional control of PGHS synthesis by glucocorticoids was described in cells such as human dermal fibroblasts (Raz *et al.,* 1989) and rat vascular cells (Pash and Bailey, 1988), and may represent

one of the universal mechanisms of PGHS regulation.

Studies employing tumour promoting phorbol esters revealed a complex pattern of PGHS regulation by protein kinase C. TPA and phorbol-12,13-dibutyrate consistently stimulated the PGE_2 output in the presence (Figs. 3-3 and 3-4) as well as in the absence (Table 3-1, and ref. 11) of exogenous arachidonate, and TPA promoted the recovery of PGHS after ASA pretreatment (Fig. 3-5). Using logarithmically transformed data, a significant stimulation of microsomal PGHS activity by TPA was also observed. These results indicate that the activation of PKC resulted in an increase of *de novo* PGHS synthesis.

Experiments in which the effect of TPA on immunoreactive PGHS levels was measured suggested the possibility that PKC activation facilitated the degradation as well as the synthesis of PGHS enzyme protein. In some tissues, such as amnion no.1 (Figure 3-6 and Table 3-1), TPA caused an increase in PGHS protein content, while in others, exemplified by amnion no.2, TPA reduced the amount of immunoreactive PGHS. Notably, amnion no.2 had a higher initial enzyme content than amnion no.1, and did not respond to indomethacin treatment with an increase in immunoreactive PGHS. This suggests that the rate of proteolytic degradation of the enzyme in this tissue was slow . Apparently, the TPA-induced PGHS degradation may lead to a decrease in the steady state PGHS protein level if the basal rate of PGHS breakdown is low. In tissues where PGHS degradation rates are higher and therefore responsive to indomethacin (eg. amnion no.1), phorbol ester stimulation may result in the accumulation of more enzyme protein. This interpretation is in agreement with the finding that indomethacin increased PGHS protein levels consistently only in the presence of the phorbol ester, i.e. where the breakdown of the enzyme was facilitated. Poor correlations between prostaglandin output, PGHS activity and protein levels were reported in PDGF stimulated mouse fibroblasts (DeWitt, 1991; Lin *et al.*, 1989),

and were attributed to a PDGF induced increase of PGHS turn over rate. The increased turnover resulted in elevated prostaglandin output while enzyme protein levels remained constant. Protein kinase C activators may regulate the PGE_2 synthesis of the human amnion by a similar mechanism.

In amnion no.2 (Fig. 3-6), a PGHS-immunoreactive band with a molecular weight slightly higher than that of the ovine standard was detected. The relationship of this material to PGHS co-migrating with the ovine standard (70 kDa), and the requirements of its production are unclear. However, in rat ovaries, a second, luteinizing hormone inducible form of PGHS was recently described, which had a M_r of 70,000 - 72 ,000 (Sirois and Richards, 1992). PGHS isoforms induced by mitogens, such as PDGF, EGF, TPA, serum, Rous sarcoma virus oncogene product, were reported in several cell lines (reviewed in ref. Xie *et al.,* 1992), and the predicted molecular weights of these proteins were all close to 70 kDa. In amnions where TPA increased the amount of PGHS protein (e.g. amnion no.1 in Fig.3-6), TPA induced PGHS - immunoreactive material with higher molecular weight than the ovine standard. Together, these observations suggest the interesting possibility that alternative form(s) of PGHS may appear in the amnion membrane under certain conditions, such as stimulation by phorbol esters or other factors *in vivo*.

The findings that TPA and cortisol do not affect PGHS activity levels to the extent that they do *de novo* synthesis rates, points to a complex mechanism of PGHS regulation. Enzyme activity values provide steady-state estimations of protein activity levels. As a result, such a measurement incorporates the effects of glucocorticoids and activators of protein kinase C on both PGHS synthesis and degradation processes. Both of these processes may be equally affected, resulting in only minor differences in observed steady-state levels following agonist treatment. In contrast, the effects of TPA and cortisol are readily

observable in studies which measure only PGHS net synthesis rates (Fig. 3-5). Taken together, these observations suggest that TPA and cortisol act to regulate PGHS through changes in enzyme synthesis and degradation rates.

In conclusion, the above experiments demonstrate that PGHS in the human amnion is positively and negatively regulated by PKC activators and glucocorticoids, respectively. Treatment of amnion tissue with these agonists changes PGHS activity levels, although perhaps at levels that may not be biologically relavent, and enzyme synthesis rates, parameters shown to change *in vivo* at labor. However, the involvement of PKC and/or corticosteroids in the *in vivo* regulation of amniotic PGHS at parturition still remains to be established.

Table 3-1

The effect of cortisol, 12-tetradecanoylphorbol 13-acetate, and indomethacin on prostaglandin endoperoxide H synthase and on PGE_2 output by the human amnion

Parameter	Amnion no.	Treatment [a] (In parentheses: lane numbers on Fig. 6) Control (2)	TPA (3)	Cort (5)	Ind (7)	TPA+ind (4)	Cort+ind (6)	Standard PGHS 2 fmol (1)	20 fmol (8)
Band intensity	1	1.0	1.5	0.83	1.1	2.2	0.89	0.45	1.4
PGE_2 output	1	136	1142	67	16	15	9	N/A	N/A
Band intensity	2	1.0	0.50	0.56	0.94	0.69	0.56	0.21	0.60
PGE_2 output	2	309	690	104	19	20	18	N/A	N/A

[a] Amnion tissue samples were treated with 12-tetradecanoylphorbol 13-acetate (TPA ,100 nmol l^{-1}), or cortisol (Cort, 100 nmol l^{-1}), or indomethacin (ind, 5 μmol l^{-1}), or vehicle (control), or the combination of 12-tetradecanoylphorbol 13-acetate and indomethacin (TPA+ind) or cortisol and indomethacin (Cort+ind) for 16 h. Proteins in the particulate (microsomal) fractions were analysed by polyacrylamide gel electrophoresis, membrane transfer, and prostaglandin endoperoxide H synthase (PGHS) immunodetection using a rabbit polyclonal antibody against ovine seminal vesicle PGHS, and an alkaline phosphatase - conjugated second antibody against rabbit IgG. PGHS (70 kDa) purified from sheep seminal vesicles was used as standard. PGHS band intensities were determined by laser densitometry and manual integration, and were expressed relative to the controls. A photograph of the immunoblots corresponding to the region of PGHS is presented on Figure 6. PGE_2 production during the agonist treatment periods was determined with RIA, and expressed as ng g^{-1} wet weight per16 h. N/A: not applicable.

3.5. Figure Legends

Figure 3-1. The effects of cortisol and RU486 on the arachidonate stimulated PGE_2 output of the human amnion.

Minced amnion tissue was incubated with increasing concentrations of cortisol in the presence (o-o) or absence (●-●) of the glucocorticoid antagonist RU486 (0.1 μmol l^{-1}) for 16 h. PGE_2 output was determined after steroid treatment by adding fresh medium with 10 μmol arachidonic acid l^{-1} for 2 h, and measuring PGE_2 with RIA. Each point is the mean of 4 parallel incubations ± SEM. Cortisol and RU486 significantly ($p<0.05$) inhibited the prostaglandin output.

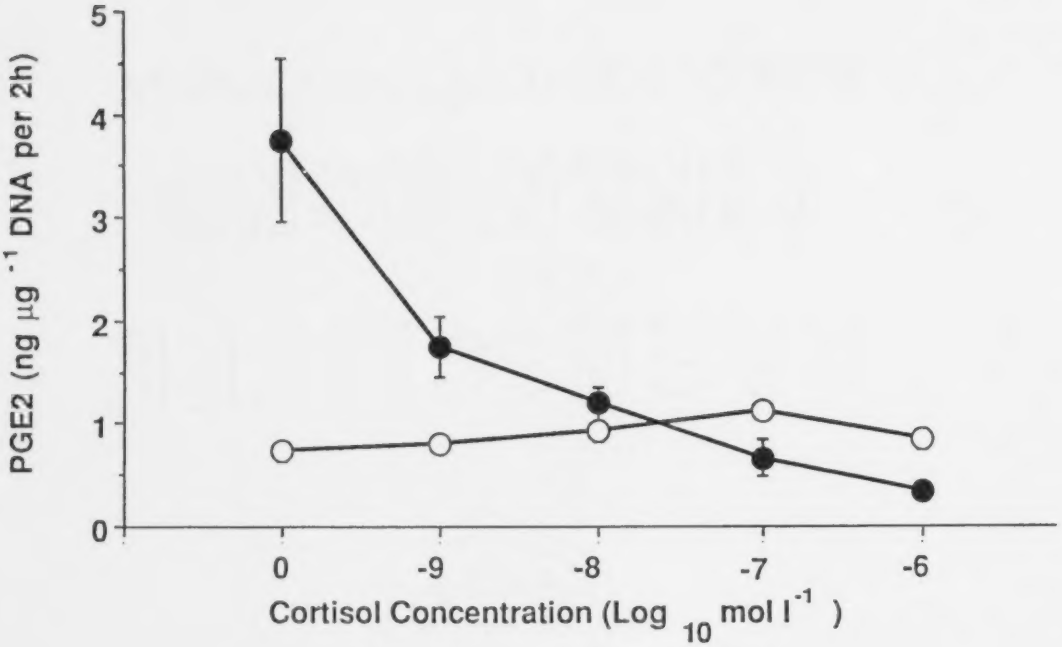

PGE2 (ng μg -1 DNA per 2h)
5
4
3
2
1
0
0
-9
-8
-7
-6
Cortisol Concentration (Log 10 mol l-1)

Figure 3-2. The effects of various steroids on the arachidonate promoted PGE_2 production of the human amnion.

Amnion tissue was incubated for 16 h with 0.1μmol l^{-1} of each of the following steroids: cortisol (CORT); RU486 (RU); dexamethasone (DEX); estradiol (E_2); progesterone (P_4); dehydroepiandrosterone sulfate (DHES); testosterone (T); 17-hydroxyprogesterone (HOP_4). Additional samples were treated with 100 μg ml l^{-1} cycloheximide (CY). Controls (C) were incubated with vehicle . Following these treatments, fresh medium with 10 μmol arachidonic acid l^{-1} was added for 2 h, and PGE_2 output was determined with RIA. PGE_2 output values, normalized to tissue DNA content, were expressed relative to the control. Each bar is the mean of results from 3-6 patients ± SEM. *: $p<0.05$ vs control (ANOVA, followed by Newman-Keuls test).

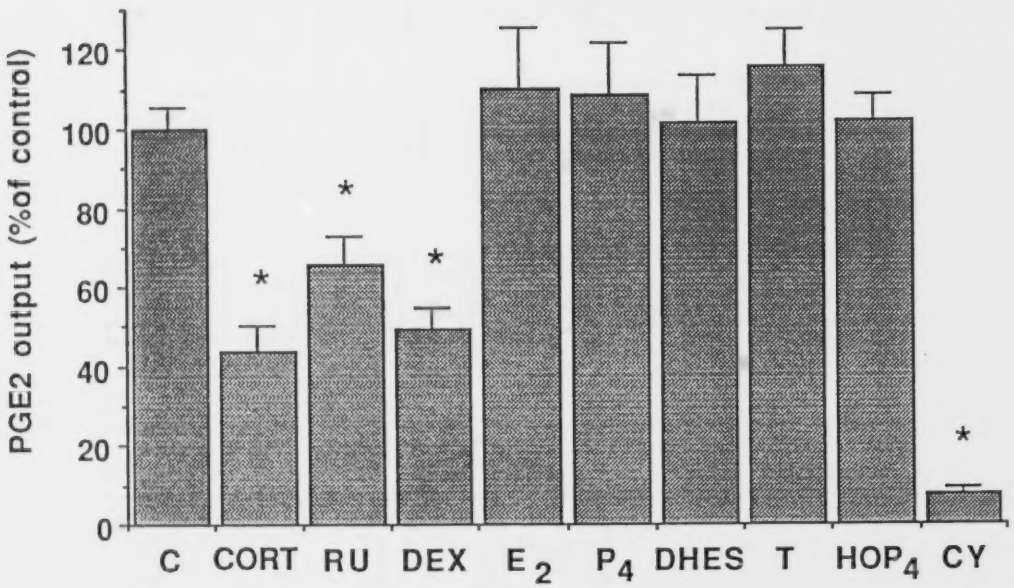

PGE2 output (%of control)
120
100
80
60
40
20
0
*
*
*
*
C
CORT
RU
DEX
E2
P4
DHES
T
HOP4
CY

Figure 3-3. The effect of TPA and cortisol on the arachidonate stimulated PGE_2 production of the human amnion.

Minced amnion tissue was treated for 16 h with increasing concentrations of the tumour promoter phorbol ester 12-tetradecanoylphorbol 13-acetate (TPA) in the presence (o-o) or absence (●-●) of 100 nmol cortisol l^{-1}. PGE_2 output was determined in subsequent 2 h incubations in fresh medium supplemented with 10 µmol l^{-1} arachidonic acid. Each point is the average of four incubations ±SEM. Cortisol significantly ($p<0.05$) decreased the TPA - stimulated PGE_2 production.

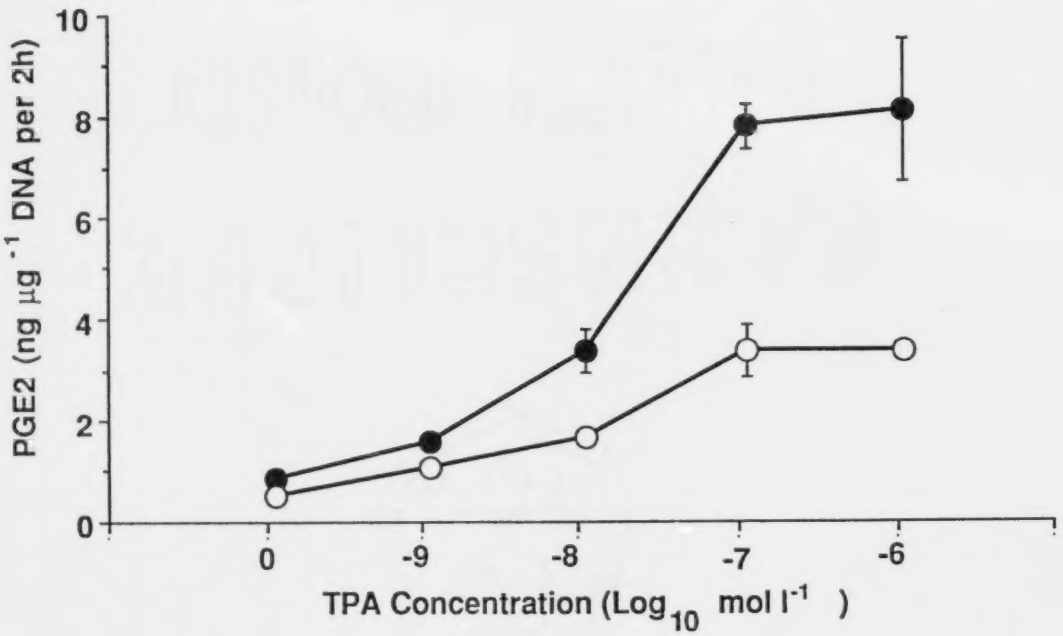

PGE2 (ng μg -1 DNA per 2h)
10
8
6
4
2
0
0
-9
-8
-7
-6
TPA Concentration (Log10 mol l-1)

Figure 3-4. The effect of phorbol derivatives on the arachidonic acid promoted PGE_2 production of human amnion tissue.

Amnion tissue samples were incubated with 100 nmol l^{-1} concentration of TPA, or phorbol-12,13-dibutyrate (PDB), or 4-methyl-TPA (4Me-TPA), or 4ß-phorbol (4ß-Ph), or vehicle (C) for 16 h. PGE_2 output was measured during subsequent 2 h incubations in fresh medium containing 10 µmol arachidonic acid l^{-1}. *: significantly ($p<0.05$) different from the control; #: significantly ($p<0.05$) less than after treatment with TPA.

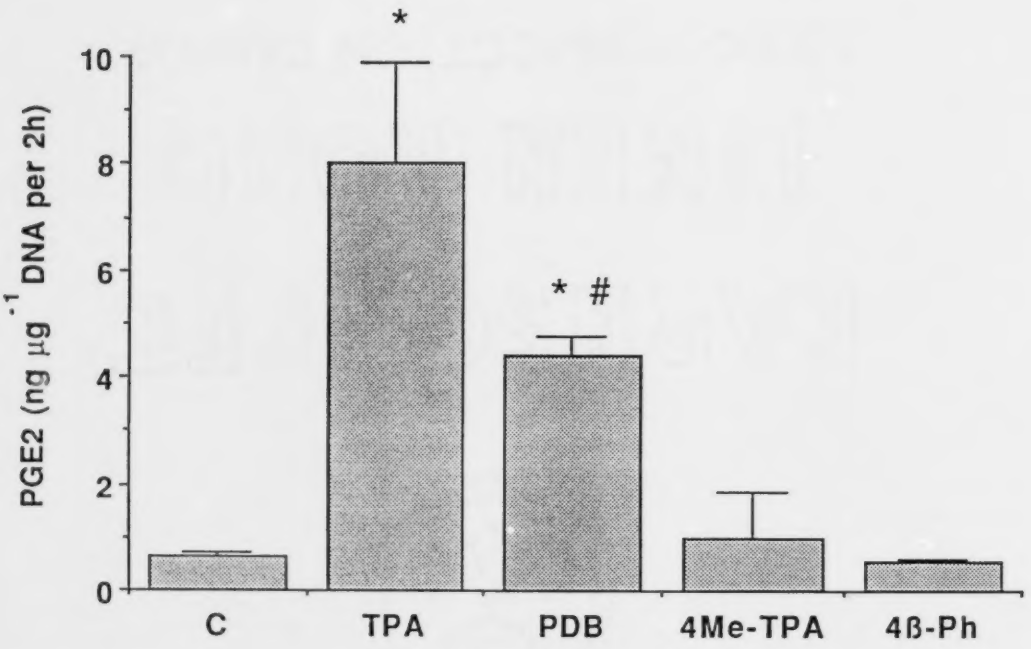

PGE2 (ng µg -1 DNA per 2h)
10
8
6
4
2
0
*
* #
C
TPA
PDB
4Me-TPA
4ß-Ph

Figure 3-5. The effect of TPA, cortisol, and actinomycin D on the recovery of PGE_2 synthesis by the human amnion after acetylsalicylic acid treatment.

Amnion tissue was incubated with 0.4 mmol l^{-1} acetylsalicylic acid (ASA) for 90 min. ASA was then removed, and the tissue samples were allowed to recover for 16 h in fresh medium with different combinations of TPA (0.1 µmol l^{-1}) cortisol (CORT, 0.1 µmol l^{-1}), and actinomycin D (Act.D, 2 µg ml^{-1}). Following recovery, new medium with 10 µmol arachidonic acid l^{-1} was added for 1 h, and PGE_2 output was determined. The efficiency of the ASA treatment to inhibit PGE_2 production was tested by adding arachidonic acid for 1 h to tissue samples not treated with ASA (no recovery, no ASA), and comparing the prostaglandin output of these samples to the prostaglandin production of tissues incubated with arachidonic acid during the last 1 h of the ASA treatment period (no recovery, ASA). Treatment combinations during the recovery interval are shown by the + signs at the bottom of the columns. Each column is the mean of four parallel incubations ± SEM. *: $p<0.05$ vs. spontaneous recovery ; #: significantly ($p<0.05$) less than after recovery in the presence of TPA.

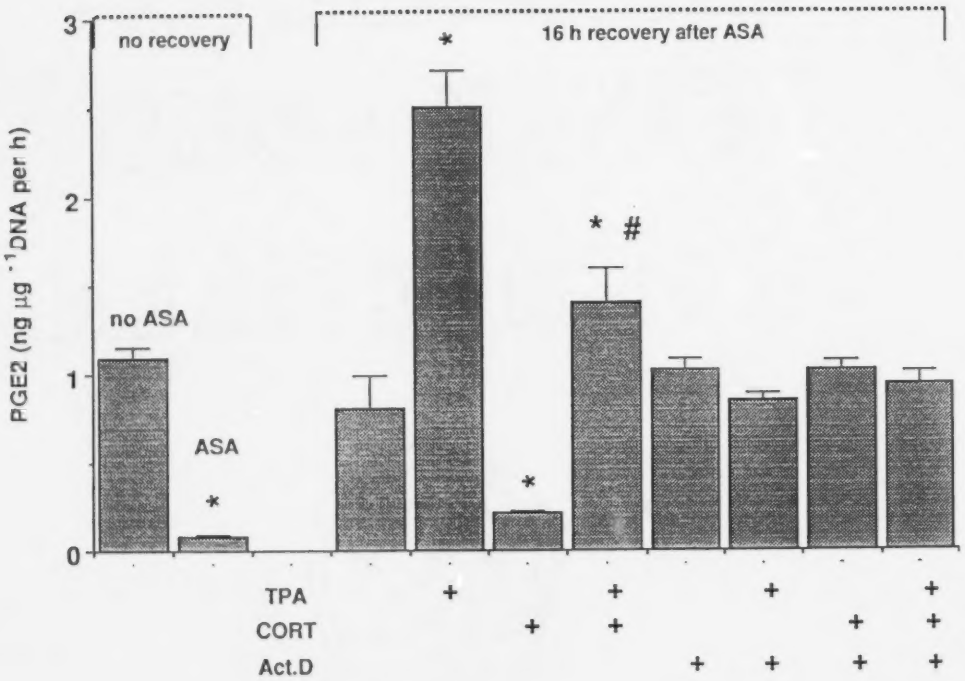
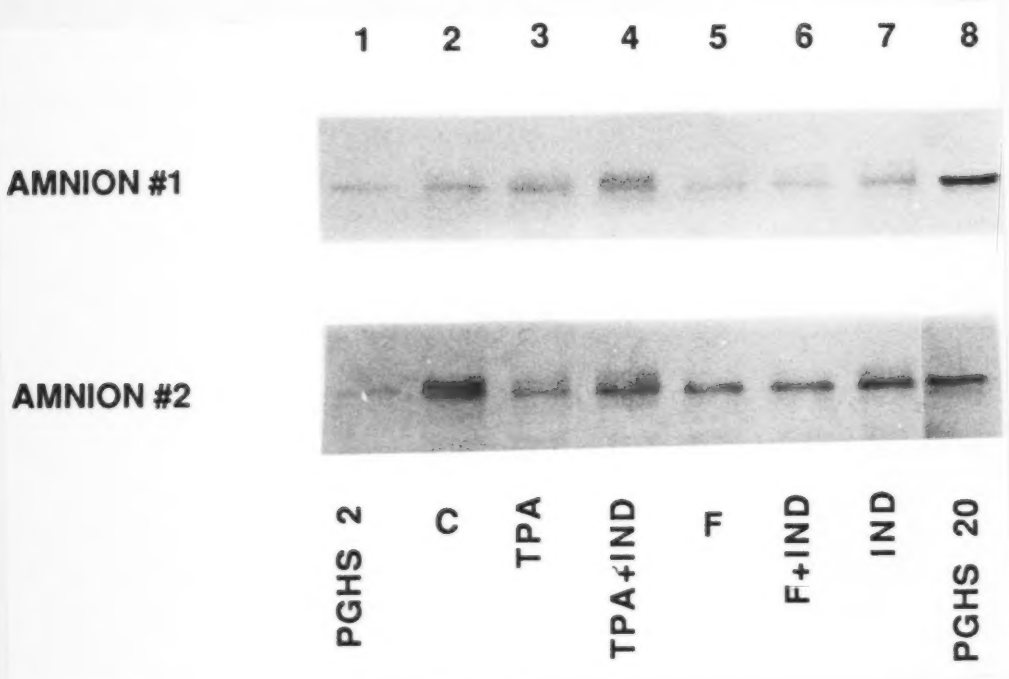

no recovery
16 h recovery after ASA
PGE2 (ng µg -1 DNA per h)
3
2
1
0
no ASA
ASA
*
*
*
* #
TPA
CORT
Act.D
+
+
+
+
+
+
+
+
+
+
+
+

Figure 3-6. The effects of TPA, cortisol, and indomethacin on the level of immunoreactive PGHS in the human amnion.

Amnion tissue samples were incubated with TPA (0.1 μmol l^{-1}), or cortisol (CORT, 0.1 μmol l^{-1}), or vehicle (CONTROL) in the presence or absence of indomethacin (IND, 5 μmol l^{-1}) for 16 h. Particulate (microsomal) fractions were prepared, and analyzed with SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to nylon membrane, and the immunoreactive PGHS protein was detected with a rabbit polyclonal antibody raised against ovine seminal vesicle PGHS, and an alkaline phosphatase-conjugated second antibody against rabbit IgG . Ovine PGHS standard (M_r: 70 kDa), 2 and 20 fmoles on lanes 1 and 8, respectively, was also run on each gel. Band intensities were determined by laser densitometry, and manual integration of the PGHS peaks. Band intensities are presented in Table 2. Lane 2: control; lane 3: TPA; lane 4: TPA+indomethacin; lane 5: cortisol; lane 6: cortisol+indomethacin; lane 7: indomethacin. The amount of protein loaded on each lane was 4 μg and 2 μg from Amnion no. 1 and Amnion no. 2, respectively.


1
2
3
4
5
6
7
8
AMNION #1
AMNION #2
PGHS 2
C
TPA
TPA+IND
F
F+IND
IND
PGHS 20

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4. Summary and Conclusions

PGs are a class of paracrine effectors that play a major role in pregnancy and parturition (Challis and Olson, 1988). Although increased PG levels in the fetal and maternal circulation as well as the amniotic fluid compartment are observed with labour onset, the mechanisms regulating PG production remain unclear. This thesis has examined the role of PGHS in controlling the production of PG in the human amnion both throughout gestation and at the time of labour. It was hypothesized that 1) increased PG production results from an increase in the specific activity of PGHS before labour at term. Similarly, 2) increased PG production at preterm labour results from increases in PGHS specific activity before labour. Further, 3) increases in PGHS activity are associated with increases in the overall amount of PGHS protein within the amnion. The mechanisms by which increased enzyme activity occurs were also examined. Thus 4) PGHS was hypothesized to be up-regulated by factors acting to stimulate the action of protein kinase C while glucocorticoids were hypothesized to inhibit PGHS within the amnion.

In chapter two, changes in PGHS activity levels were examined at both term and pre-term labour in the human amnion. Although similar studies have been performed to examine the changes in PGHS enzyme activity at term, the data presented herein are the first to document increases in PGHS activity at pre-term labour. In accordance with the first two hypotheses, significant increases in PGHS activity were observed at both term and pre-term labour. A reasonable point of contention arising from these results may be the question of causality. Were the observed increases in PGHS activity occurring before labour and thus possibly causal to labour or were they the result of labour initiation and thus a consequence of labour? In an attempt to answer this question, a cross-sectional analysis was made of amnion tissue samples collected across gestation, prior to

the onset of labour. These data clearly demonstrate an increase in PGHS activity prior to the onset of labour in the days and hours immediately preceding the expected time of term labour onset. This occurs at a time when PG levels are also known to be increasing (Kierse, Mitchell and Turnbull, 1977). These observations are consistent with those of Smieja *et al.* (1993) who postulated that term C/S groups of patients fall into arbitrarily defined high and low PGHS activity groups. Those with a high PGHS activity value would presumably be closer to labour onset than those patients observed to have a low PGHS value at the time of obstetrical intervention. The inference from this observation is that increases in PGHS must occur rapidly prior to labour. Our results are consistent with this supposition. Care must be taken however in concluding outright that increased PGHS activity is causal to human labour. The definition of the not-in-labour C/S group is crucial in defining labour onset. In these studies, the definition of non-laboured amnion samples was taken as those tissues collected at elective C/S in which the patients had no cervical dilatation, uterine contractions, induction or augmentation. These criteria are clinically-based and thus may not be indicative of the subtle biochemical changes that define labour onset such as increased gap junction formation (MacKenzie and Garfield, 1985). Indeed, Casey and MacDonald (1993) propose that the initiation of human parturition involves a transition from uterine quiescence (phase 0) to uterine preparedness for labour (e.g. gap junction formation), phase 1, to phase 2, in which active labour results from increases in uterotonic agents such as PG and oxytocin. Given this, it must be remembered that, in the human, ethical considerations preclude definitive studies to establish the exact time or mechanism of labour onset.

The etiology of preterm labour onset is multifactorial. In many situations, intrauterine infection and the associated inflammatory response are known to cause premature rupture of membranes and ultimately labour (Romero *et al.*,

1988). However, a significant proportion of all pre-term deliveries arise from idiopathic pre-term labour. By measuring the specific activity of PGHS both before and after preterm labour in these uninfected tissues, an increase in PGHS activity was observed similar to that found in term labour tissues. While the same questions of cause and effect arise, this is the first study to document increases in PGHS activity in association with preterm labour. Although not specifically examined, a connection between elevated PGHS activity and infection-associated pre-term labour may lie at the level of increased PGHS expression. Cytokines, paracrine factors released by inflammatory cells, are known to stimulate PG production by eliciting the expression of PGHS-2 in confluent cultures of amnion cells (Mitchell *et al.*, 1992). The tissues used in this study were histologically confirmed to be free of infection. In this case, infection was defined as neutrophil invasion of the intrauterine membranes. However, in the decidua, there are resident cells that are bone-marrow derived and thus, may share similar properties to the related macrophages. It may be that local cytokine production by these cells in the decidua stimulates adjacent amnion tissue to increase PGHS activity in a manner identical to that described above. Under such circumstances, pre-term labour would result without any clinical indications of infection.

This thesis has also attempted to explain the mechanism(s) by which PGHS activity is increased in the human amnion. PGHS is known to have a high turnover rate (Fagen and Goldberg, 1986). The enzyme is continually inactivated during PG synthesis and it is estimated that 1 pmol of PGHS can synthesize 1300 pmol of PG before inactivation (Marshall, Kulmacz and Lands, 1987). As a result, continuous synthesis of the PGHS protein is required for sustained PG production. Furthermore, increased steady-state levels of PGHS activity require higher rates of PGHS enzyme synthesis; and it is reasonable to hypothesize that

an increased enzyme synthesis rate results in higher levels of enzyme protein in the tissues. However, the absence of any correlation between PGHS activity and immunoreactive protein levels infers that irPGHS protein concentrations do not reflect PGHS activity levels in amnion tissue. Most likely, amnion microsomes contain varying amounts of enzymatically inactive, undegraded PGHS protein in addition to the active enzyme. Since our tissue collecting and processing protocol preserves active PGHS, we conjecture that most of the inactive pool of PGHS was formed *in vivo*, during enzyme catalysis. Therefore, enzyme activity levels can be expected to correlate more closely with amount of freshly synthesized PGHS than with total irPGHS. Thus, metabolic labeling with ^{35}S-Methionine and immunoprecipitation studies of newly formed enzyme should be performed to establish a correlation between enzyme synthesis rates and activity levels.

The presence of both PGHS-1 and -2 isoforms in the amnion may also account for the lack of correlation between enzyme activity and protein levels. Although the anti-sheep PGHS polyclonal antibody used in the immunoblotting experiments recognize both isoforms, it may be that the immunoreaction is stronger with one PGHS isoenzyme than with the other. This may result in variations in signal intensity depending on the relative amounts of the two proteins present in the microsomal preparations. As well, the rapidity at which PGHS-2 is induced and subsequently degraded may also explain the lack of correlation between enzyme activity and protein levels. In contrast to the constitutive PGHS-1 message and protein, PGHS-2 is known to be transiently expressed in response to agonist stimulation. The mRNA for PGHS in the mouse contains 12 Shaw/Kamen sequences inferring a sort half-life for this message (Xie, Robertson and Simmons, 1993). The human PGHS-1 mRNA, in

contrast, has none. The presence of PGHS-2 protein in only 36% of the samples is consistent with the transient nature of the expression of this isoform.

The mechanisms responsible for PGHS induction *in vivo* were not specifically tested, although treatment of amnion tissue with TPA was shown to increase both the amount of irPGHS and the synthesis rate of PGHS. These results are consistent with the fourth hypothesis that activators of protein kinase C stimulate PGHS in the amnion. Studies in chapter 3 demonstrate that TPA acts to increase PGE_2 output and the synthesis rate of PGHS. At the same time, TPA had little effect on the steady-state levels of PGHS activity. Thus, TPA may elicit increases in PGHS synthesis rates (Fig. 3-5) that would, in turn, lead to an increase in PG output and the accumulation in irPGHS protein (Fig. 3-6, amnion #1). The lack of change in the tissue level of PGHS enzyme activity following TPA treatment is suggestive of a concomitant increase in PGHS inactivation. Stimulation of enzyme synthesis as well as degradation results in an increased enzyme turnover rate with enhanced product formation and little or no alteration in steady-state activity levels. In tissues containing relatively large amounts of PGHS, synthesis rates may already be maximally stimulated and the addition of TPA would predominantly affect PGHS degradation (Fig 3-6, amnion #2).

It was further hypothesized that glucocorticoids would act to inhibit PGHS in the amnion. Our findings that glucocorticoids do indeed inhibit PG output, PGHS synthesis and, to some extent enzyme activity, are consistent with those of Gibb and Lavoie (1990) who demonstrated a decrease in PGE_2 output following dexamethasone treatment in freshly dispersed amnion cells. In vascular smooth muscle cells, glucocorticoids also act to suppress PG production by inhibiting PGHS mRNA levels (Bailey, Makheja and Pash, 1988). Glucocorticoids may act to inhibit the transcription of PGHS mRNA which would ultimately lead to a decrease in PG production, PGHS protein and, to a smaller

extent, enzyme activity levels. The results from chapter 3 would point to a role for glucocorticoids in regulating PGHS turnover rates. Cortisol was found to markedly inhibit the *de novo* synthesis of PGHS but had little effect on steady-state PGHS activity levels in the microsomes. Thus, decreases in PGHS synthesis rate may be accompanied by a decrease in PGHS inactivation and degradation resulting in very little change in overall PGHS steady-state levels as measured by the enzyme assay. Inhibition of arachidonate release by glucocorticoids (Potestio and Olson, 1990) could account for a decrease in PGHS inactivation rate.

Therefore, in conclusion, increases in PGHS activity have been documented both in the case of term and pre-term labour. These increases are observed prior to the onset of labour and thus may be causal to the known increases in PG levels before the onset of labour in humans. The mechanisms responsible for these increases are not completely defined. However, the results would indicate that factors such as changes in enzyme synthesis and degradation rates play an important role in the regulation of PGHS. As well, the detection of both isoforms of PGHS in the amnion may indicate a pathway of rapid enzyme induction through *de novo* protein synthesis at labour. Endogenous factors found within the intrauterine compartment such as cortisol and activators of protein kinase C (e.g. diacylglycerol) may contribute to PG production by acting at the level of PGHS and thus regulating arachidonate metabolism. The overall result is an increase in the synthetic capacity of the amnion to produce the uterotonic agents responsible for the passage of the fetus through the birth canal.

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Appendix 1: Release Form

Permission is hereby granted to Fernando Jose Teixeira to include the manuscript "Regulation of Prostaglandin Endoperoxide H Synthase by Glucocorticoids and Activators of Protein Kinase C in the Human Amnion" as chapter 3 of the thesis "The Role and Regulation of Prostaglandin Endoperoxide H Synthase in Human Labour." Mr. Teixeira is second author on this publication and made contributions to this study by performing enzyme assays and Western blot analyses of PGHS.

Tamas Zakar

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Appendix 2: Solutions and Buffers

40% Acrylamide:	38.8 g Acrylamide 1.2 g Bis-acrylamide Make up to 100 ml with distilled water
4x Running Buffer:	12.11 g Trizma base 57.6 g Glycine Make up to 1 L with distilled water Adjust pH to 8.2- 8.3
10x Transfer Blocking: Solution (TBS)	292 g NaCl 250 ml of 250 mM Tris-HCl Make up to 1 L with distilled water
2x Sample Loading Buffer:	50 ml of 0.5 M Tris-HCl 20 ml glycerol 20 ml of 10% SDS 5 ml of 2-Mercaptoethanol 2 ml of 1% Bromphenol blue 3 ml of Distilled water
Alkaline Phosphatase Buffer:	25 ml of 1 M Tris-HCl 25 ml of 0.5 M $MgCl_2$ 25 ml of 1 M NaCl 175 ml of Distilled water
Blocking Solution:	1x TBS containing 5% non-fat dry milk
Citrate Buffer:	50 mM Citrate Make up to 500 ml with distilled water Adjust pH to 3.0 Supplement with 15% (v/v) ethanol

Cofactor Mixture:	15 ml of Incubation buffer 10.95 g of Reduced glutathione 28. 50 g of Tryptophan
Homogenisation Buffer:	50 mM Tris HCl 2 mM EDTA 0.25 M Sucrose Make up to 500 ml with distilled water Adjust pH to 8.0
Incubation Buffer:	50 mM Tris HCl 2 mM EDTA Make up to 500 ml with distilled water Adjust pH to 8.0
Phosphate Buffered Saline with Gelatin:	10.76 g of Sodium phosphate (monobasic) 32.7 g of Sodium phosphate (dibasic) 18.00 g NaCl 2.0 g Sodium Azide 2.0 g Gelatin Make up to 2 L with distilled water Adjust pH to 7.1
Pseudoamniotic Fluid:	13.2 g NaCl 0.6 g KCl 3.89 ml of $CaCl_2$ (2.05 M) 0.96 ml of $MgCl_2$ (2.90 M) 0.16 g Sodium phosphate (dibasic) 9.53 g HEPES acid 740 mg Urea 720 g Dextrose 200 mg Bovine serum albumin Make up to 2 L with distilled water Adjust pH to 7.2

Running Buffer:	10 of 10% SDS
	250 ml of 4x Running buffer
	740 ml of Distilled water
Transfer Buffer:	1 L of 4x Loading buffer
	3 L of Distilled water
	1 L Methanol

Appendix 3: Validation Experiments

The experiments contained within this appendix are validation studies for the enzyme assay and western blot portion of the thesis. Although not specifically included in chapters 2 and 3, the results are pertinent for an understanding of the data presented within those chapters. The methods employed for Figures A3-1 through A3-5 are described in detail in chapter 2 with the relevant modifications described in the individual legends.

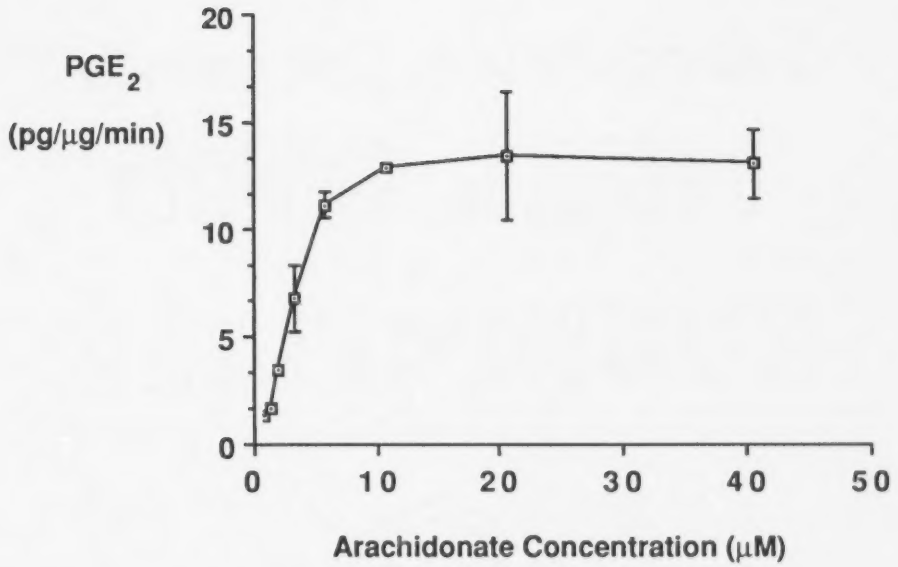

Figure A3-1: Saturation of PGHS by increasing concentrations of arachidonic acid. Amnion microsomes were prepared as described in chapter 2. Increasing concentrations of arachidonate were incubated in the presence of enzyme co-factors and microsomes for 4 minutes. The prostaglandin E_2 produced was extracted and measured by a specific radioimmunoassay. The data were then normalized for microsomal protein content. Results are expressed as the $\bar{x}$ ± S.D. of four individual data points and are representative of three separate experiments performed on three individual amnions.

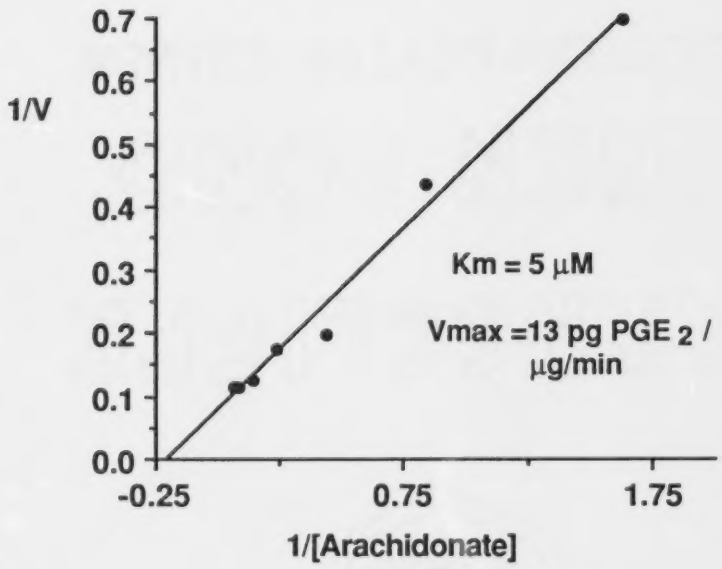

Figure A3-2: Saturation of PGHS by increasing concentrations of arachidonic acid using Lineweaver-Burk analysis. Amnion microsomes were prepared as described in chapter 2. Increasing concentrations of arachidonate were incubated in the presence of enzyme co-factors and microsomes for 4 minutes. The prostaglandin E_2 produced was extracted and measured by a specific radioimmunoassay. The data were then normalized for microsomal protein content. Results are expressed as the $\bar{x}$ of four individual data points. The abscissa is expressed as the inverse of the arachidonate concentration while the ordinate is plotted as the inverse of calculated PGE_2 output/μg protein/min. The K_m and V_{max} values of 5 μM and 13 pg PGE_2/μg protein/minute respectively were determined using simple linear regression from data inputed to the Cricket Graph computer programme.

Comparison of V_{max} and Enzyme Activity at 20 μM Arachidonate

	Activity at 20 μM (pg PGE_2/μg protein/minute)	Vmax	p
C/S (n = 4)	16.5 ± 4.0	16.6 ± 3.6	p = 0.96
SL (n = 4)	53.0 ± 17.2	57.8 ± 17.8	p = 0.71

Table A3-1: Comparison of PGHS specific activity (at 20 μM arachidonate) and V_{max}. Amnion microsomes were prepared as described in chapter 2 from a cesarean section and spontaneous labour placenta. The tissues were processed as described in Figure A3-1. Each tissue was processed on four individual occasions in order to study interassay variation. Data are expressed as the $\bar{x}$ ± S.D. The calculated V_{max} was compared to PGHS specific activity at 20 μM arachidonate using a Student's t-test. The was no significant difference in the activity of the enzyme at 20 μM arachidonate and the calculated V_{max}.

PGHS Enzyme Assay Precision

	Mean (pg PGE_2/μg protein/minute)	STD. DEV.	C.O.V.
C/S (n = 4)	16.6	3.6	21%
SL (n = 4)	57.8	17.6	30%
Sheep (n = 10)	36.0	11.7	32%

Table A3-2: Calculation of PGHS enzyme assay precision. Tissue samples from individual amnions collected from a single cesarean section and spontaneous labour patients and an ovine lung sample were processed as described in chapter 2. PGHS enzyme activity, as measured in pg PGE_2/μg protein/minute, was determined on either four (C/S or SL) or ten (sheep) separate occasions and the $\bar{x} \pm$ S.D. determined. The coefficient of variation (C.O.V.) was calculated using the equation C.O.V. = Std. Dev./Mean. The calculated C.O.V. for the spontaneous labour amnion was used to determine the initial sample size for the studies carried out in chapter 2.

Appendix 4: Statistical Analysis for PGHS Enzyme Assay

The data presented herein are the grouped means of six separate amnions following experimental treatment as described in Chapter 3 of the thesis.

	Treatment:	Control	Cortisol	TPA
Amnion #:	1	91.2	55.6	59.5
	2	84.4	75.5	57.4
	3	49.1	63.5	46.6
	4	8.2	8.3	15.9
	5	11.1	15.7	50.0
	6	9.2	6.3	13.8

(Data expressed in pg PGE_2/μg/4 minutes)

Analysis of Variance:

Source	DF	SS	MS	F	P
Factor	2	69	34	0.04	0.965
Error	15	14283	952		
Total	17	14351			

Treatment	N	Mean	Standard Deviation
Control	6	42.2	38.58
Cortisol	6	37.48	30.82
TPA	6	40.53	20.46

Curriculum Vitae

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Research Experience:
1989-1990: Undergraduate Research Student, Lawson Research Institute, University of Western Ontario (Supervisor: Dr. D.M. Olson).

1990-1991: Graduate Student, Department of Physiology, University of Western Ontario (Supervisor: Dr. D.M. Olson).

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Teaching:
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Honours and Awards:
1986: University of Western Ontario Entrance Scholarship, University of Western Ontario.

1987-1988: Captain, University of Western Ontario Varsity Soccer Team.

1990: Dean's Honour List, University of Western Ontario.

1990: Lawson Research Institute Studentship (salary).

1992: Mary Louise Imrie Graduate Award, University of Alberta.

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Invited Presentations:

1990: "The Effects of Developmental Age on Prostaglandin-Mediated cAMP Production in Perinatal Rat Fibroblasts." Seminar: Lawson Research Institute "Try on For Size" Seminar Series (May), University of Western Ontario.

1991: "Regulation and Ontogeny of Prostaglandin H Synthase in the Human Amnion." Seminar: Lawson Research Institute "Try on For Size" Seminar Series (May), University of Western Ontario.

1992: "Cyclooxygenase Activity in Human Amnion Throughout Gestation." Seminar: Department of Obstetrics and Gynaecology Research Day (May), University of Alberta.

1992: "Prostaglandin Endoperoxide H Synthase (PGHS) Activity Increases with Gestation and Labour in Human Amnion." Eighth Annual International Conference on Prostaglandins and Related Compounds, Montreal, Quebec (Vol 8: p 89, Abs #343) (Selected for Perinatal Plenary Session) (July, 1992)

1993: "Prostaglandins and Parturition: A Causal Role in Human Labour." Seminar: Perinatal Research Centre Seminar Series (May), University of Alberta.

1993: "The Role of Prostaglandins in Human Labour Initiation.": Seminar: Department of Obstetrics and Gynaecology Research Day (May), University of Alberta.

Publications:

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Hirst JJ, ***Teixeira FJ***, Zakar T, Olson DM. Prostaglandin Endoperoxide H Synthase-II mRNA and Immunoreactive Protein Levels in Human Amnion and Decidua. Third European Congress on Prostaglandins In Reproduction. Edinburgh, Scotland, August, 1993 (in press).

Activities:

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1990: Vice-President and Founding Member, Physiology Students Association, University of Alberta.

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1992: Tutorial Coordinator, Undergraduate Physiology, University of Alberta.

1992: Member, Student's International Health Association: Africa '93 Project, University of Alberta.

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